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FINAL TECHNICAL REPORT
STUDY OF THE FUNDAMENTAL PRINCIPLES OF BIO-ELECTROCHEMISTRY

by

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ABSTRACT

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Reactions of biological systems at an electrode have been investigated experimentally and through theoretical considerations to determine the capability of biochemical reactions in producing useful electrochemical energy. Experimental work with micro-organisms and several enzymes established that electrochemical energy may be derived either through formation of enzymatic conversion of electrochemically inactive fuels to active products or from electron exchange between the electrode and the enzyme via an electron carrier intermediary molecule. However, no evidence was obtained for a significant electron transfer directly from enzymes to the electrode. Were such a reaction to exist, theoretical calculations for a model system suggest that the maximum current through the direct reaction would be very small in relation to the output possible from the other types of reactions.

Laboratory studies also demonstrated the need for extreme care in defining the actual reactive electrochemical systems in the biofuel cells since extraneous materials in the biological systems may be responsible for relatively large, temporary electrical outputs.

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SUMMARY

A program of studies has been carried out on the fundamental aspects of electrochemical activity in biological systems. It has been directed to the following specific tasks:

(1) Survey of enzyme and bacterial systems for identification of those showing electrochemical activity.

(2) Study of those systems identified under 1 for determination of the molecular species involved in the electrode reaction.

(3) Theoretical analysis of the problem of the reaction of biological macromolecules at electrodes. Determination of the theoretical maximum value of current to be expected from such reaction.

(4) Experimental study of the electrode behavior of model redox macromolecules containing active groups of known redox characteristics. The objective of this work was to ascertain the aspects of behavior related specifically to the low diffusivity and special orientation of macromolecules.

(5) Study of methods of attaching or coating biological systems, including enzymes and living bacterial cells, onto electrode surfaces. Effectiveness and behavior of systems so attached were studied.

(6) Investigation of the electrochemical efficiency of bio-electrode systems including energy and material balances, and the factors affecting current and voltage characteristics.

The major findings which have resulted from the program are the following:

(1) In all systems studied, any electrochemical activity observed was shown to be due either to nonbiological substances present as impurities or additives, or to nonbiological substances formed as intermediates or products of the biological reaction.

(2) Special studies designed specifically to demonstrate the direct reaction of biological macromolecules at electrodes gave no evidence of such reaction.

(3) Theoretical treatment of the direct reaction of biological macromolecules at electrodes indicated that maximum electrode currents obtainable from such reactions are not large, even under the most favorable assumptions.

SECTION 1

INTRODUCTION

The objectives of this program have been to carry out a study of the basic processes involved in the generation of electrical energy by biological systems. The approach which was adopted was, first, to determine the classes of biological systems capable of electrochemical activity. These were then to be subjected to detailed study in order to define the specific reactions involved in the electrochemical process.

An associated objective was to study the more pragmatic aspects of the generation of electrical energy by living bacterial systems. Particularly, it was desired to examine factors such as theoretical efficiency of bioelectrodes, the performance of electrodes with attached versus unattached organism cultures, and the viability and growth of bacterial cultures in the presence of electrodes.

In accordance with these objectives the program has been directed to study of the electrochemical activity of a number of enzyme systems including D-amino acid oxidase, urease and glucose oxidase, etc. Also included were studies on several bacterial systems, both attached to the electrode and suspended in solution, including B. subtilis, Proteus vulgaris, and suspensions of cell mitochondria. Electrochemical activity was found in many of these. However in all such cases, the activity was shown to be primarily due either to electroactive impurities initially present in the materials used to make up the biological system, or to the formation of low molecular weight electroactive substances as intermediate or final products of the reaction between the biological agent and its substrate. It is these substances which provide the electrode reaction.

Recent thinking as to possible applications of bio-electrochemical phenomena, including the generation of practical quantities of electrical power, is, to an increasing degree, encountering the question of direct participation of biological macromolecules or bacterial cells in oxidation-reduction processes at electrodes. Accordingly, the latter part of the program placed increased emphasis upon determination of whether or not such direct participation ever occurs.

Initial approach was to define, on the basis of theoretical considerations, the conditions under which direct participation of biological molecules might be observable. Subsequent work was then concerned with attempts to observe such participation under the theoretically most promising conditions. A promising but unfinished part of the program was the study of the electrochemical behavior of synthetic macromolecules which correspond to biological macromolecules in size and structure, but which have attached groups of known oxidation-reduction characteristics.

These latter studies have suggested that the development of practical quantities of electrical energy from the direct reaction of biological macromolecules or cells is not promising. However, the work of the program, as described in the following sections, can be regarded only as the initial phase in the development of understanding of this very interesting field.

SECTION 2

MATERIALS AND METHODS

2.1 MATERIALS. SOURCES AND PREPARATIONS

a. Micro-organisms

From American Type Culture Collection; Proteus vulgaris (13315), Streptococcus faecalis (8013), Bacillus subtilis (6051), Endomyces decipiens (Reese) (11647). Escherischia coli is a wild type strain, subcultured from samples obtained originally from University of California, Los Angeles.

b. Enzymes

Certain enzymes were prepared by Philco Research Laboratories to obtain quantities of enzyme in higher purity than that available commercially. These included: D-amino acid oxidase, crystalline and precrystalline, from hog kidneys by the method of Massey (1). Purification was carried to a precrystalline state (ca 0.5 U/mg* protein) or to crystalline material (ca 12 U/mg) depending upon the use requirements. Urease, crystalline; from jack bean meal, was prepared by the method of Hanabusa (2) with activity in the range of 11,000 to 13,00 Sumner units**

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* Unless otherwise specified, an enzyme unit (U) refers to the amount of activity required to perform the conversion of one micromole (μmole) substrate to product per minute.

** A Sumner unit corresponds to the formation of 1 mg ammonia nitrogen per 5 minutes from urea under specified conditions.

per mg protein. Sulfite-nitrite reductase (SNR)*, $NADPH_2$ specific, was obtained from E. coli by slight modifications of the procedure of Kemp, et al (3).

Enzymes obtained from commercial sources: DAO from hog kidney, about 0.05 U/mg, catalase, purified, 8250 U/mg from Sigma Chemical Company; fungal glucose oxidase (15 U/mg), yeast alcohol dehydrogenase (2x crystallized, 18 U/mg), diaphorase (from Clostridium kluyveri, 10 U/mg) and rabbit muscle lactic dehydrogenase (2x crystallized, 31.3 U/mg) from Mann Research Laboratories; Urease, 380 Sumner units per mg, from California Corporation for Biochemical Research; L-arginase (beef liver, 20 U/mg) and L-glutamic dehydrogenase (from E. coli, 0.6 U/mg) from Worthington Biochemical Company.

The sugar oxidases from Iridophycus flaccidum (4) and from orange fruits (5) were prepared at the University of California, Riverside.

c. Coenzymes, Substrates and Chemicals

All chemicals including substrates and coenzymes were obtained from commercial sources: from Sigma Chemical Company, the D-amino acids, alanine, tryptophan, phenylalanine, tyrosine methionine, valine, leucine, and (allo)-isoleucine and the coenzymes ATP, NAD, and $NADPH_2$; from Calbiochem, FAD, phenylpyruvic acid (sodium salt), and p-hydroxyphenyl pyruvic acid; from K & K Laboratories, D-histidine, methylene blue, 2,6-dichlorophenol indophenol and indole pyruvic acid.

Other chemicals were obtained as reagent grade materials from various sources. The Folin-Ciocalteau Phenol reagent for protein determinations was a concentrated solution obtained from Uni-tech. Materials for bacterial media were from Difco.

2.2 METHODS

a. Growth and Storage of Micro-Organisms

For usual growth of bacteria, standard techniques were used; growing in appropriate media for the given organism, inoculating from a starting culture into 500 ml erlenmeyer flasks and shaking (for aerobic species) at 37°C until the

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* Abbreviations used in this report include: NADP, Nicotinamide adenine dinucleotide phosphate (Coenzyme II or pyridine trinucleotide); $NADPH_2$, reduced NADP; NAD, nicotinamide adenine dinucleotide; $NADH_2$, reduced NAD; ATP, Adenosine tri-phosphate; FAD, flavine adenine dinucleotide; DAO, D-amino acid oxidase; SNR, sulfite-nitrite reductase; IPA, indole pyruvic acid; PPA, phenyl pyruvic acid; HPA p-hydroxyphenyl pyruvic acid; ma, milliampere; μa, microampere; SCE, saturated calomel electrode; g, gravities.

desired optical density (600 mμ, turbidometric measurement) was obtained; and harvesting by centrifugation. For pellicle formation, B. subtilis was grown without shaking. Harvested cells were used rapidly in bioelectrode measurements or stored after freeze-drying. Drying was accomplished by suspending the cell paste in 0.5 percent ascorbic acid, 2.0 percent ammonium chloride, pH 7.0, shell freezing at dry ice temperature and drying in vacuo while maintaining the temperature of the suspension at -26°C.

For preparation of the E. coli in sufficient amounts for SNR enzyme preparation and for larger bioelectrode studies, an 8 liter fermenter was inoculated with the starter culture and the bacteria were grown at 37°C until the desired turbidometric measurement was obtained. In the SNR preparation, the bacteria were adapted to growth on C medium (6), a medium containing only inorganic salts with glucose as the carbon source. The bacteria were freeze-dried after growth and kept until a sufficient amount of material had been accumulated for carrying out the preparative work.

b. Enzyme Preparations

Published procedures were used for enzyme preparations (see Materials section) except for SNR. In this case, it differed from the published method only in the extractive procedure. E. coli, freeze-dried, were ground while cold, in a mortar and then incubated, as per published procedure, with ribonuclease and deoxyribonuclease. Grinding of the dried cells took the place of the published procedure of disrupting a fresh, frozen cell paste in a Hughes press.

Mitochondria were obtained from fresh, cooled rat liver with normal isolation techniques using 0.25 M sucrose containing 1 percent serum albumin as the suspending medium and a Waring blender disintegration. Initial mitochondrial pellets, obtained at 5000 x g, were washed twice, centrifuged at 24,000 x g and finally dispersed in the original medium. Mitochondria were also prepared from cauliflower with slight modifications of the method of Crane (7).

Activity determinations involving oxygen uptake were performed by the Warburg micro-manometric techniques, using standard procedures. For mitochondrial activity determinations, succinate was used as substrate. DAO activity was determined manometrically using D-alanine as substrate, with a pH 8.3, pyrophosphate buffer. The various glucose oxidase preparations were assayed manometrically with appropriate buffers for optimal activity (pH 5.6 for fungal and citrus preparations and pH 6 for the algal enzyme) with glucose substrate.

Urease activity was also determined manometrically by observing the release of carbon dioxide at pH 5.0. Assays were also performed using a micro-Nesslerization procedure for the release of ammonia at pH 8.3.

Alcohol dehydrogenase was assayed by determination of the rate of reduction of NAD as indicated by the increase in optical density at 340 mμ. Ethanol was used as substrate, at pH 8.5. Lactic acid dehydrogenase was similarly determined, using lactate at pH 7.4. SNR was determined spectrophotometrically by the decrease in optical density at 340 mμ using $NADH_2$ with the oxidant substrates nitrite, sulfite or hydroxylamine.

Diaphorase activity was determined by following the rate of reduction of 2,6-dichlorophenol indophenol by the enzyme and $NADH_2$ at pH 7.5.

The method of Lowry, et al (8) was employed for most protein determinations. The biuret reaction (9) was occasionally used when the conditions interfered with the Lowry method.

c. Polarization Measurements on the Bioelectrode

Most polarization measurements were conducted with the apparatus schematically illustrated in Figure 1. Potential between test electrode, e_1 and reference electrode, e_2 (SCE), was maintained at the desired level (usually +0.200 v) by adjusting the variable resistance R to obtain the necessary polarizing current. The value of 0.200 volt was selected since differentiation between individual systems seemed to be greatest at this level. In some experiments, an automatic potentiostat (Anotrol) replaced the manual system.

The polarization cell used for the majority of the experiments is shown in Figure 2. Slight modifications were made in the illustrated design to allow for continuous passage of gas and the addition of constituents to the cell as well as withdrawing samples during a run. Temperature was maintained at 38°C by circulating constant temperature water through the outer jacket.

For experiments in which material was limited or in which high concentrations of enzymes were to be maintained in the vicinity of the electrode, the cell pictured in Figure 3 was utilized. Stirring in this cell was accomplished through vibration of a stirring bar at 60 cps with amplitude controlled by a variable transformer power supply to the vibrating motor. In some studies the electrode compartment was separated by a semipermeable membrane from the remainder of the cell. In this cell the electrode was also vibrated to provide stirring within its own compartment.

The electrode material used in almost all the experiments was bright platinum. This was selected to avoid problems in determining effective electrode surface and uncontrolled adsorption effects. In some experiments a platinized (platinum black) electrode was used. These are designated in the following discussions.

When anaerobic experiments were being done, the test solutions in the cell were deaereated by bubbling nitrogen gas (prepurified and further de-oxygenated by passing over hot, reduced copper) through the solution prior to addition of the enzyme.

Buffer solutions used to maintain pH during the course of the reaction were normally 0.1 M or higher, thus providing adequate ion concentration for maintaining relatively low cell resistances.

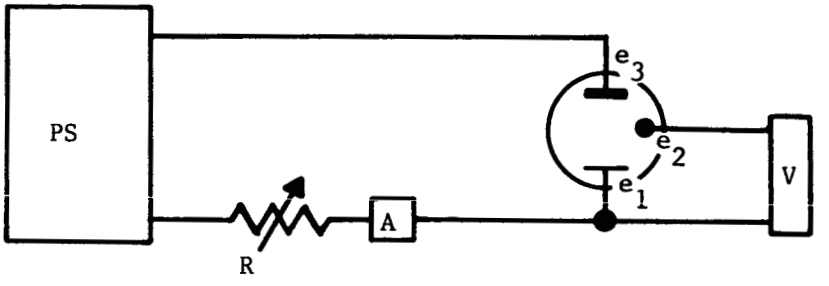

FIGURE 1. SCHEMATIC DIAGRAM OF APPARATUS USED FOR POLARIZATION MEASUREMENT OF BIO-ELECTRODE

PS = HEWLETT-PACKARD 711A D.C. POWER SUPPLY, A = SIMPSON 269 MICROAMMETER VOM, V = HEWLETT-PACKARD 412A VACUUM TUBE VOLTMETER, R = INDUSTRIAL INSTRUMENT DECADE RESISTANCE BOX, e_3 = AUXILIARY ELECTRODE, e_2 = REFERENCE ELECTRODE (SCE), e_1 = TEST ELECTRODE (PLATINIZED PLATINUM DISC)

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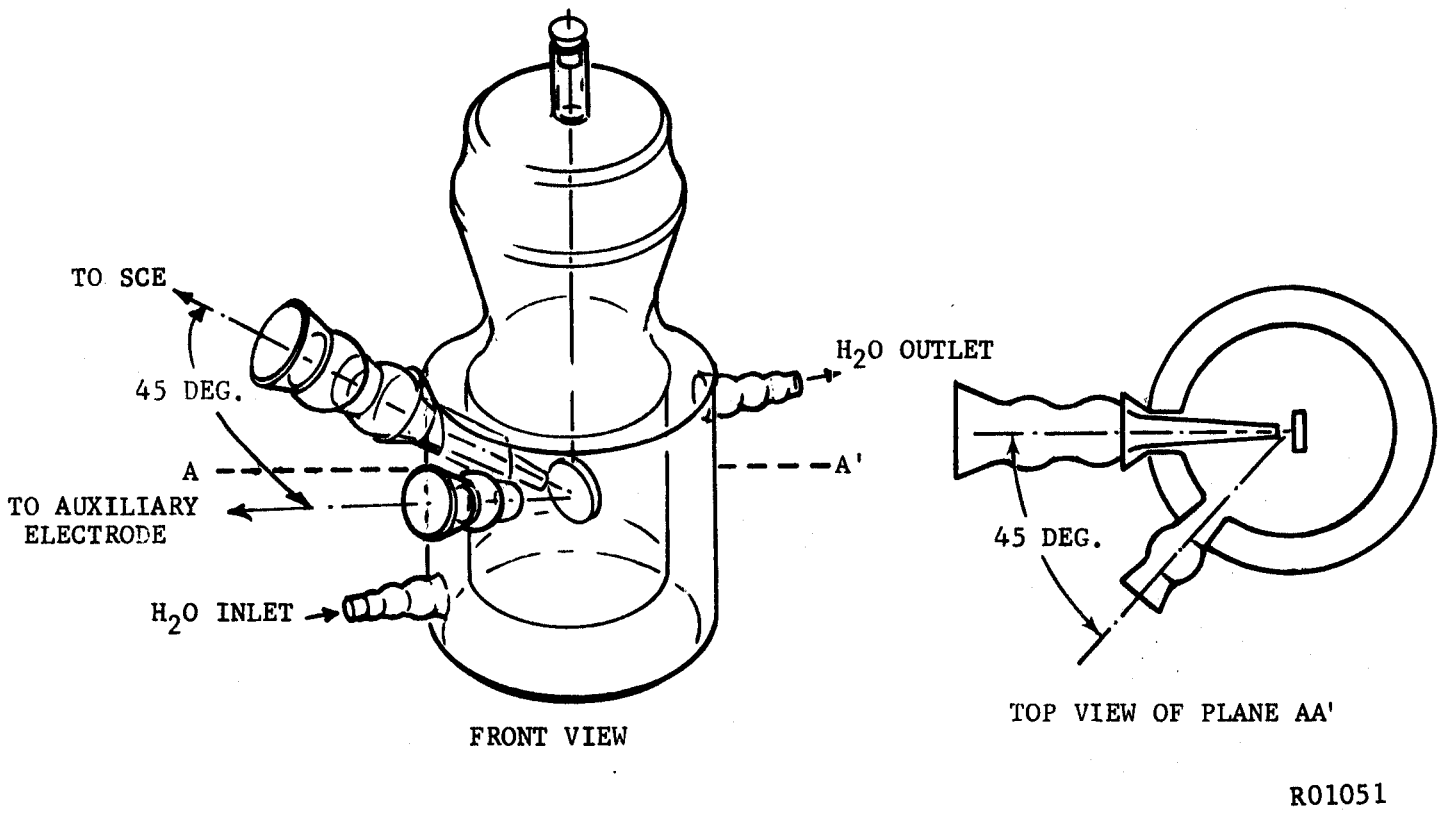
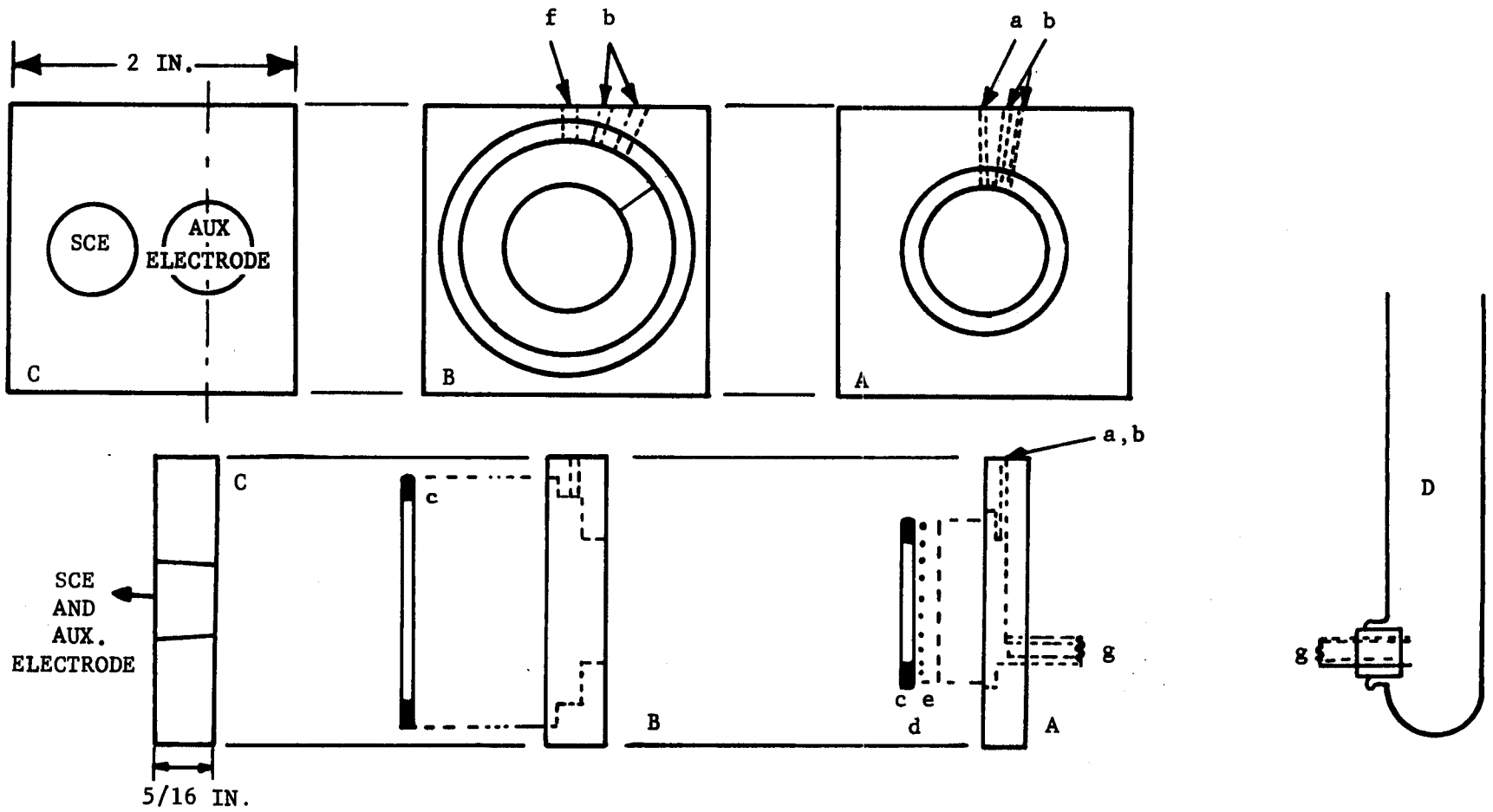


FIGURE 2. POLARIZATION CELL WITH FRONT SIDE LUGGIN-HABER CAPILLARY



A - ELECTRODE COMPARTMENT UNIT
- a. ELECTRODE SHAFT APERTURE
- b. FILLING AND GASSING APERTURES
- c. SILICONE GASKET
- d. SUPPORT SCREEN
- e. SEMIPERMEABLE MEMBRANE

B - FUEL CHAMBER
- f. STIRRER APERTURE

C - BACK PLATE

D - SCE TUBE
- g. TEFLON CONNECTING TUBE WITH SALT-AGAR BRIDGE

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FIGURE 3. MICROELECTROCHEMICAL CELL WITH SEPARATE ELECTRODE COMPARTMENT

d. Chronopotentiometric Analysis of Bioelectrode Reactions

The principle of chronopotentiometry is based upon constant-current, electrolysis in unstirred solutions. A thorough theoretical discussion of the significance of chronopotentiograms has been given by Delahay and co-workers (10) and by Reinmuth (11 a, b). Much valuable information on the mechanism and kinetics of chemical and electrochemical reactions may be obtained from analysis of the transition time (τ) and the potential-time curve.

The experimental studies were performed with the instrument represented in Figure 4. The cell is connected to a voltage regulated power supply PS (Hewlett-Packard 711A) through a large series resistor R_1 which insures a constant current to the cell. The test electrode (e_1) potential is measured against the reference electrode e_2 (SCE) by means of recorder R (Mosely Autograf X-Y recorder, Model 2DR-2). A preamplifier P having an input impedance of more than 100 megohms is used in the circuit between the test electrode and reference to minimize current flow between these electrodes. With resistor R_2, current to be used for electrolysis may be preselected. Resistor R_2 is adjusted so that when DPDT switch is thrown from position S_1 to S_2 substantially no change in current is observed on ammeter A (Simpson 269 Microammeter VOM). Prior to electrolysis, current is passed through R_1 and R_2 until these resistors reach thermal equilibrium. The pen time drive on the recorder is then started, simultaneously throwing the DPDT switch from position S_1 to S_2 and the potential-time curve is recorded. Not shown in this diagram, was a switch inserted in the circuit to reverse the polarity of electrodes e_1 and e_3 so as to enable use of the electrolysis for either oxidative or reductive electrode reactions.

Electrolysis was conducted in a cell similar to that in Figure 2. Anaerobic conditions were normally used. The test electrode was a smooth platinum disk (ca 1/4 inch in diameter) sealed in the end of a soft glass tube. Electrical contact was through a nickel wire welded to the back of the electrode. This electrode was inserted into a larger diameter glass tubing which acted as a mantle. The mantle extended approximately one inch below the surface of the test solution. When measurements were made in unstirred solution the electrode was withdrawn about 1/4 inch into the mantle; in stirred solution the electrode extended slightly beyond the mantle. The experiments were done with the electrode in "reduced", "oxidized" or "clean" states as defined below.

An "oxidized" electrode refers to an electrode which was brought to the potential of oxygen evolution. "Reduced" electrode is one which has been reduced to the potential of hydrogen evolution. Some of the experiments with biological material made it apparent that another classification of treatment was essential since it was discovered that some materials coat the electrode in such a way that it no longer behaves as a normal platinum electrode after normal reduction or oxidation of the surface electrolytically. To obtain a "clean" electrode, it was treated by anodizing and cathodizing alternately in 50% nitric acid solution, cathodizing in sulfuric acid (ca 1N), rinsed in water, wiped dry and placed in the sample solution and reduced chronopotentiometrically to hydrogen evolution potential Similar results could usually be obtained with the less drastic measure of vigorous hydrogen evolution in acid. One of these three treatments was used on the electrode prior to each potentiogram.

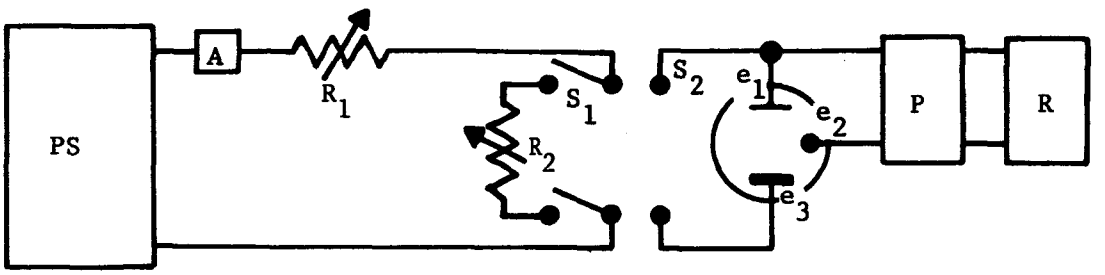

FIGURE 4. SCHEMATIC DIAGRAM OF APPARATUS USED FOR CHRONOPOTENTIOMETRY

PS = HEWLETT-PACKARD 711A D.C. POWER SUPPLY, A = SIMPSON 269 MICROAMMETER VOM, R_1 AND R_2 = CLAROSTAT VARIABLE RESISTORS, P = PREAMPLIFIER, R = MOSELEY AUTOGRAF X-Y RECORDER, MODEL 2DR-2, e_1 = TEST ELECTRODE, e_2 = REFERENCE ELECTRODE (SCE), e_3 = AUXILIARY ELECTRODE, S_1 AND S_2 = POSITIONS OF DPDT SWITCH

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Chronopotentiometric measurements were normally performed anaerobically by flushing with nitrogen as described in 2-2C. Since preparing the "clean" electrode required withdrawing the electrode from its mantle and great precautions were essential in maintaining true anaerobicity of some solutions (particularly DAO-tryptophan) nitrogen flushing of the mantle was carried out during the removal, period of treatment and return of the electrode.

d. Other Procedures

Spectrophotometric measurements made use of either Cary Model 14 or Beckman DK-2A recording spectrophotometers. Electrophoresis was performed with Research Specialties Company equipment.

SECTION 3

EXPERIMENTS AND RESULTS

3.1 SURVEY OF ENZYME REACTIONS FOR SUITABLE ANODE REACTIONS

Although a number of enzymes had been previously tested for suitability in bioanode reactions, the objectives and different approaches used in the present investigation made it essential to carry out a relatively extensive survey of enzyme reactions in the polarization experiments to define the best systems for study. The major effort was devoted to investigation of the oxidative enzymes, but, since the hydrolytic enzyme urease was reported to give positive bioanode effects, a few other hydrolytic enzymes were also tested. In the latter cases, the results helped in establishing the basis for the observed electrochemical activity of urease.

One of the major objectives of the survey was to detect any possible direct reaction between the enzyme concerned and the electrode. Thus, the use of electron carrier mediators such as ferricyanide or methylene blue was not stressed in these studies. However, the effect of such materials upon the electrochemical behavior of the system studied was frequently determined.

3.2 HYDROLYTIC ENZYMES

a. Arginase

The power production in the urease reaction has been attributed in the past to the electrochemical oxidation of the ammonia liberated in the reaction (12, 13) with some implications that the electrode reaction involved an "activated" intermediate formed in the biological reaction. If such were the case similar activated complexes or intermediator might be found in other hydrolytic reactions. Arginase hydrolyses arginine to urea and ornithine. Tests of this enzyme demonstrated that it was completely inactive electrochemically. It should be noted for

later discussion that the reaction here does not involve a significant change in pH during the hydrolytic reaction.

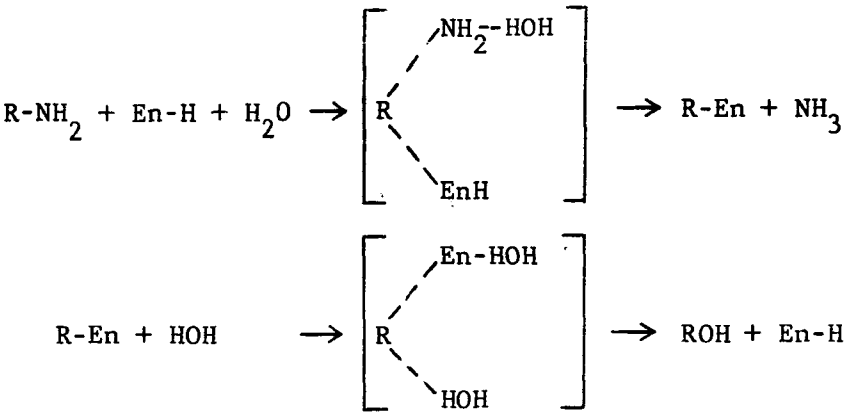
b. Amino acid decarboxylases

The amino acid decarboxylases are widely distributed in bacteria, generally forming in adaptive response to a specific amino acid. The reaction for these enzymes is $RCHNH_2COOH \longrightarrow RCH_2NH_2 + CO_2$, essentially a hydrolytic splitting of the carbon to carbon bond. During the reaction of these enzymes, unless carried out in adequate buffering medium, a pH change may occur.

Tests of glutamic decarboxylase of E. coli showed no significant activity in the electrochemical cell.

c. Urease

Urease, catalyzing the reaction, $CO(NH_2)_2 + H_2O \longrightarrow 2\ NH_3 + CO_2$, has been a dilemma in the bioelectrochemical field. The reaction occurs through the mechanism related to general hydrolytic reactions.



In the case of urea, ROH would be the intermediate carbamide which is subsequently hydrolyzed to NH_3 and CO_2 by a second reaction sequence similar to that shown. There is no significant electron redistribution during the enzyme action nor is any intermediate formed which would be expected to have significant electrochemical activity under physiological conditions. Yet, a number of sources (12, 13) report significant amounts of current and power obtainable from the action of this enzyme on urease in an electrochemical cell. Normally, one would not expect to have a significant electrochemical oxidation of ammonia at the pH of the optimal conditions for enzyme activity and survival and this presumption is borne out in tests of the oxidation of ammonia as illustrated in Figure 5. In a cell with a bright platinum electrode, no significant difference in behavior is observed between cell solution containing buffer alone, and buffer plus ammonia. However, the action of urease upon urea appears to give significant increase in current as shown for commercial urease preparations in Figure 6 and for a crystalline urease preparation in Figure 7.

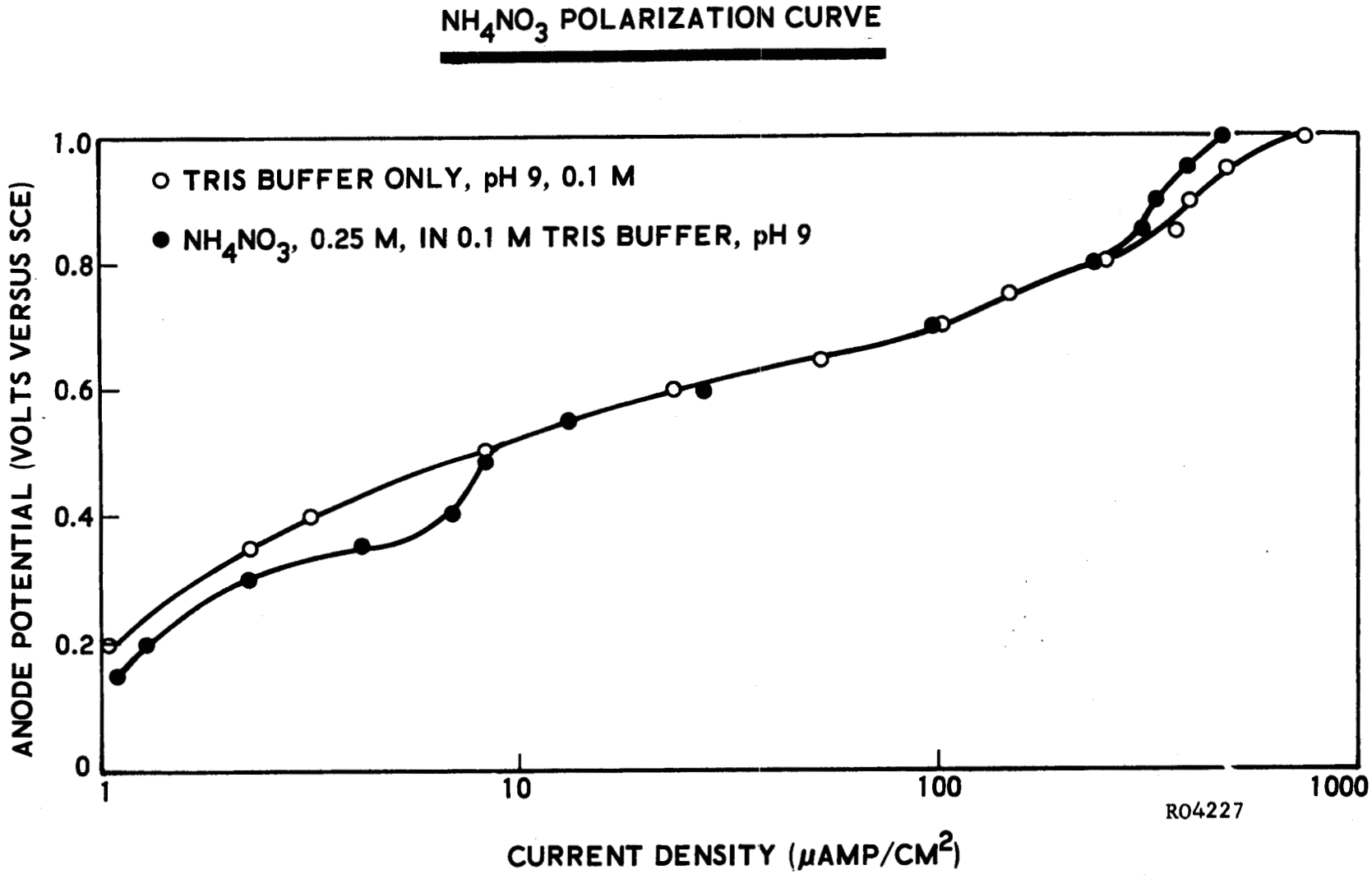


FIGURE 5. NH_4NO_3 POLARIZATION CURVE

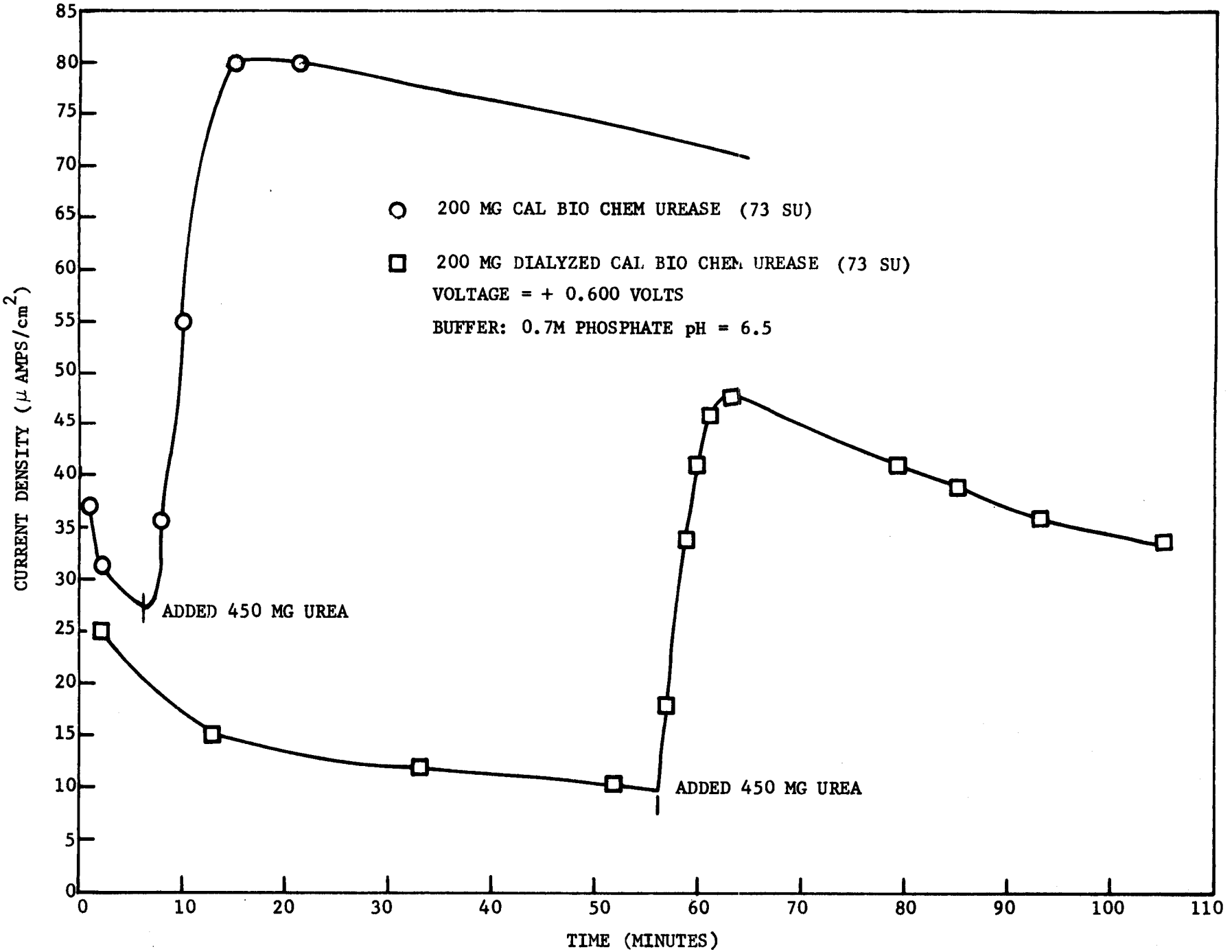


FIGURE 6. ELECTROCHEMICAL ACTIVITY OF COMMERCIAL UREASE - UREA SYSTEM

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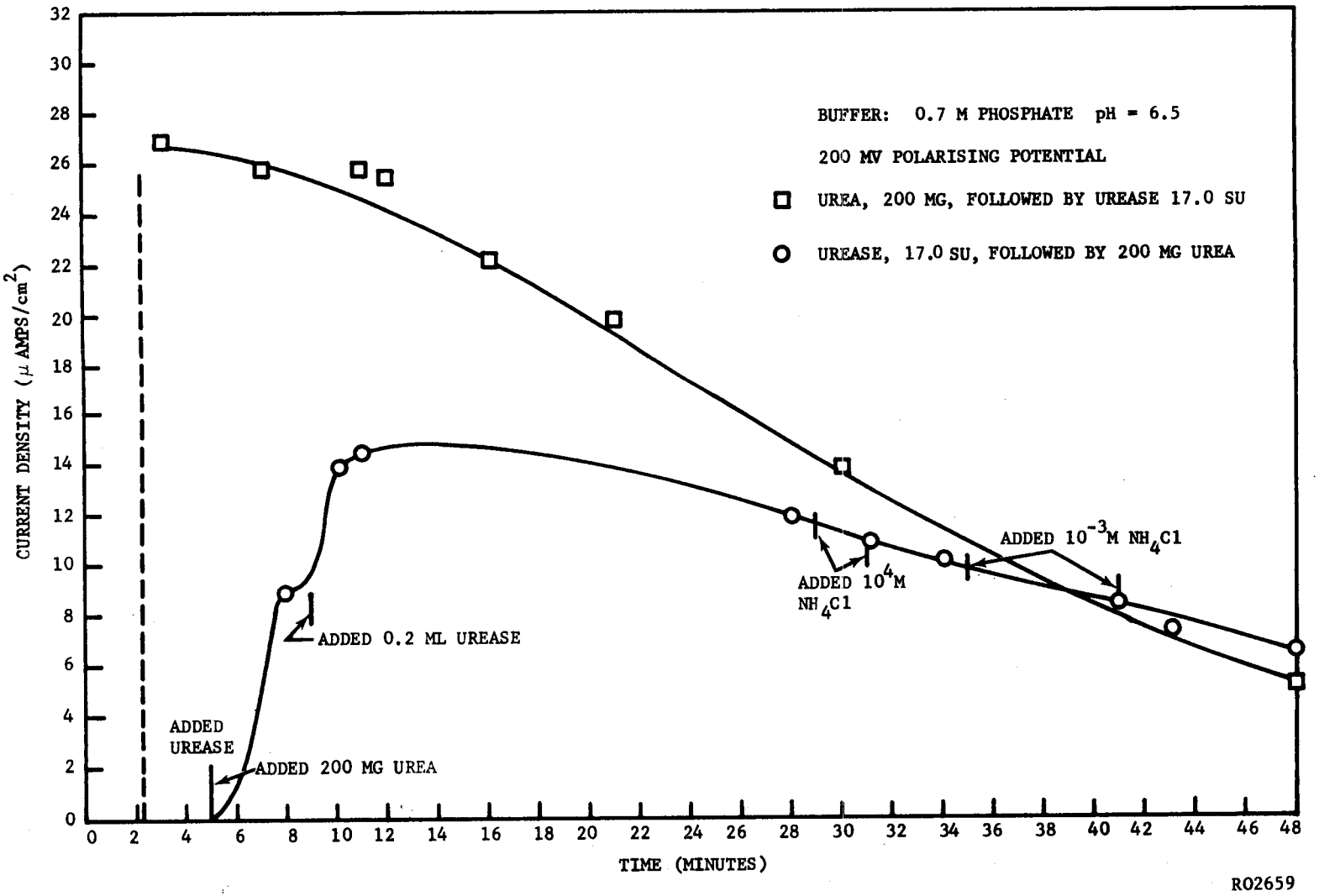

FIGURE 7. ELECTROCHEMICAL ACTIVITY OF UREASE WITH UREA

It became evident in early work that the pH had a profound effect upon the results obtained with urease as, for example, is demonstrated in Figure 8. The difference between commercial enzyme preparations and purified enzyme is also evident from comparison of Figures 6 and 7. It was possible to prove that the commercial preparations of enzyme contained several kinds of electrochemically active material by dialysis experiments. Samples of urease were dialyzed against limited quantities of buffer and the dialyzing buffer was retained for subsequent determination of electrochemical activity along with the dialyzed enzyme. As shown in Figure 9, the electrochemical activity of such a purified urease-urea system is much reduced from that found in the nondialyzed preparation (Figure 6) and both the first and second dialyzing buffers contain large amounts of electroactive material. However, the purified enzyme is seen to retain electrochemical activity.

The current obtainable with the urea - urease system is observed to be directly proportional to the extent of hydrolysis (Figure 10). In the experiment shown here it became evident that hydrolysis was occurring considerably more rapidly than had been expected. Under these circumstances it seemed possible that the extent of reaction might have resulted in breaking through the buffering capacity of the medium. A test of pH change and current production with the commercial enzyme showed a direct parallel between rise in pH and rise in current (Figure 11). In a confirming test to establish the effect of pH, a change in current was obtained similar to that found in the normal urease-urea system, simply by adding sufficient alkali to the urease alone to induce the same pH change which normally occurs upon the hydrolytic reaction (Figure 12).

A final test of the effect of pH was made through the use of a buffer of maximum strength (2.5 M, pH 6.5). No change in current was observed during the hydrolytic reaction nor was there any significant pH change in this experiment.

Further tests of purified urease, rather than the commercial preparation, demonstrated that it was possible by dialysis to remove impurities of low molecular weight to eliminate background currents totally. No change in current would then be observed during hydrolytic reactions. These tests all indicate that any electrochemical activity obtained from the reaction of urease upon urea must be due to the increase in pH as a result of hydrolysis which labilizes to electrochemical oxidation some of the impurities normally found in the urease preparations.

3.3 OXIDATIVE ENZYMES

Of the oxidative enzymes tested, only one, D-amino acid oxidase, showed initial results compatible with a direct participation of enzyme in the electrode reaction. Accordingly, the greatest effort was expended in characterization of the reaction responsible for this effect. As will be demonstrated in the following paragraphs, even for this system the power was obtained through an indirect mechanism.

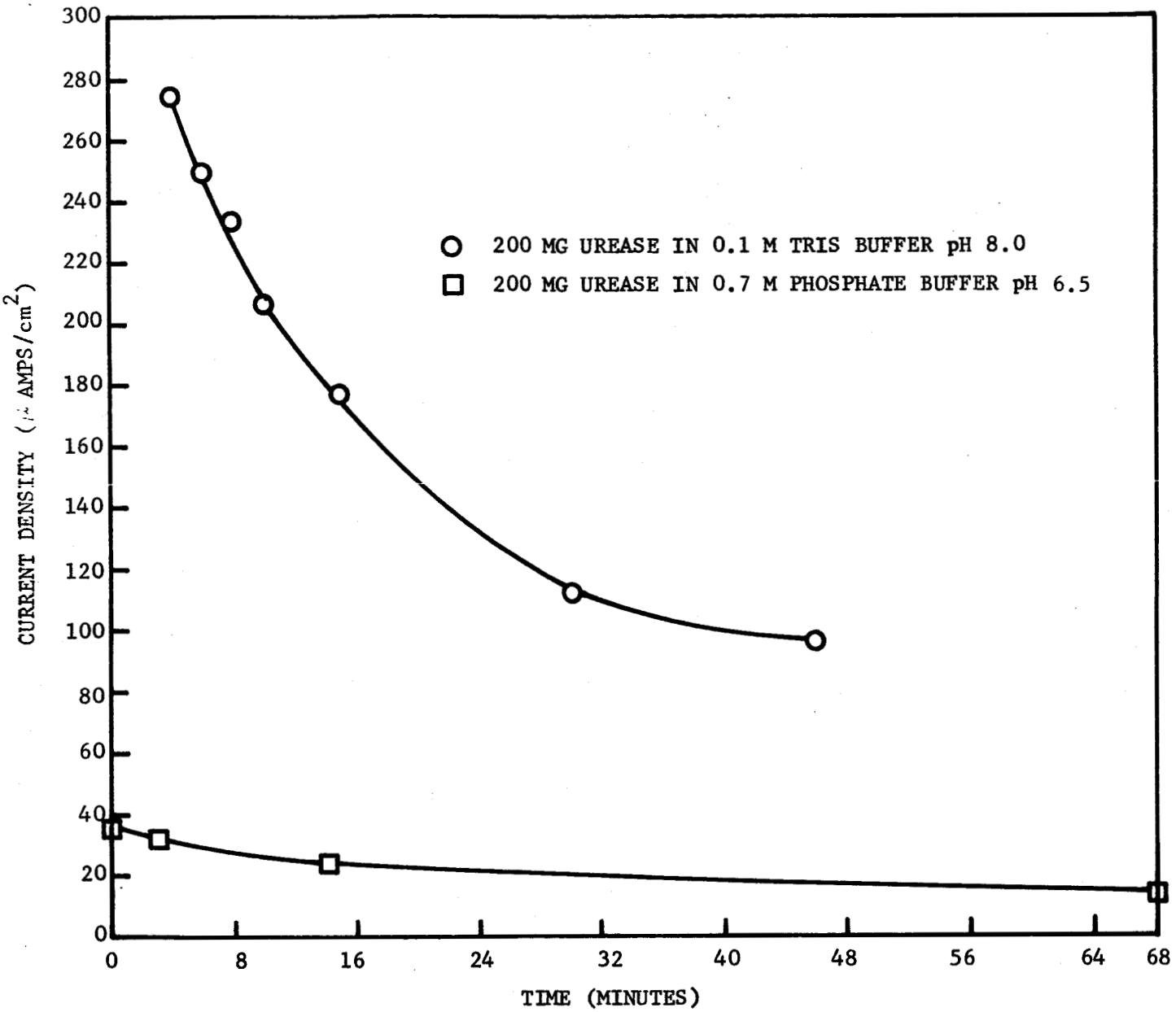

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FIGURE 8. EFFECT OF BUFFER AND pH ON CURRENT FROM UREASE

CONDITIONS: UREASE. COMMERCIAL, 333 MG IN 50 ML. pH 6.5, 0.1 M PHOSPHATE BUFFER, DIALYZED AGAINST 50 ML BUFFER TWICE, 48 HR (A) AND 2.5 HRS (B) 30 ML OF DIALYZED ENZYME (U) OR DIALYZING MEDIA (A OR B) USED IN ELECTROCHEMICAL CELL TESTS. 450 MG UREA ADDED AT INDICATED POINTS. ANODE POTENTIAL 0.6 V VERSUS SCE.

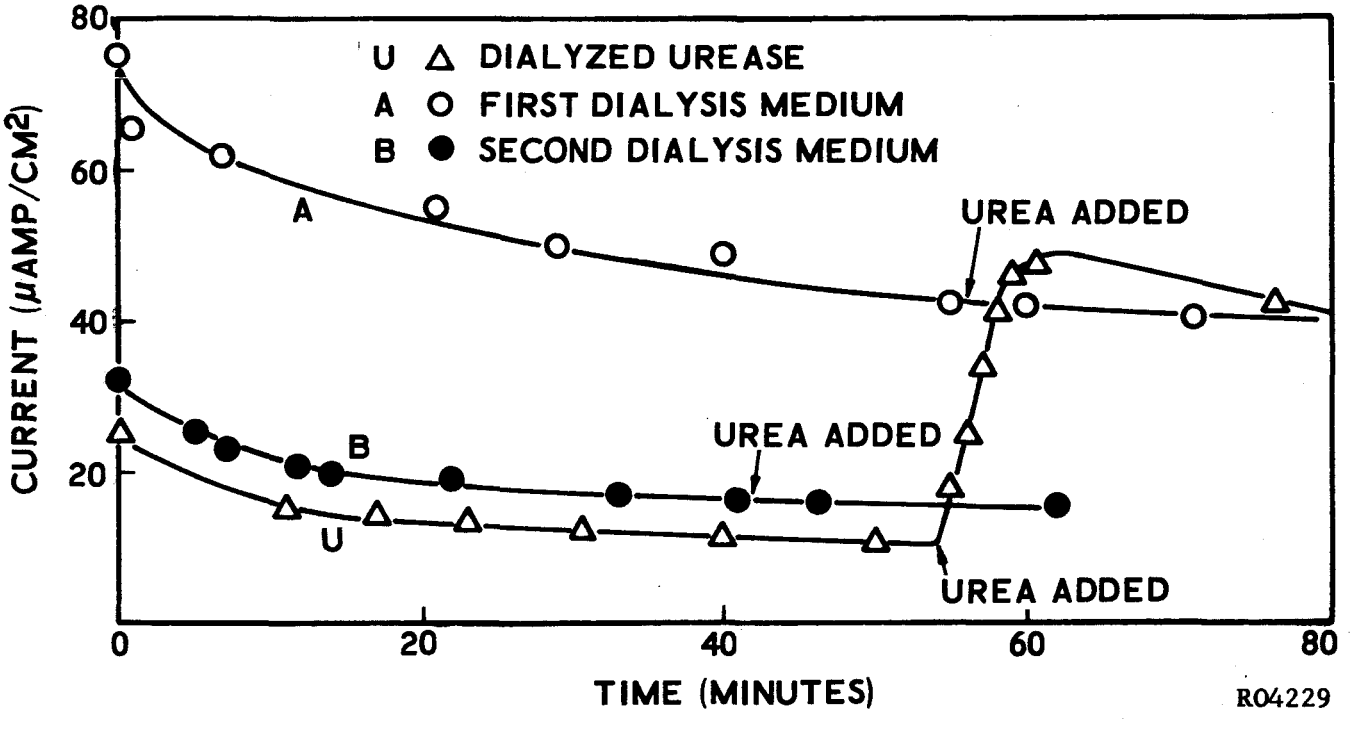

FIGURE 9. EFFECT OF DIALYSIS ON CURRENTS FROM COMMERCIAL UREASE

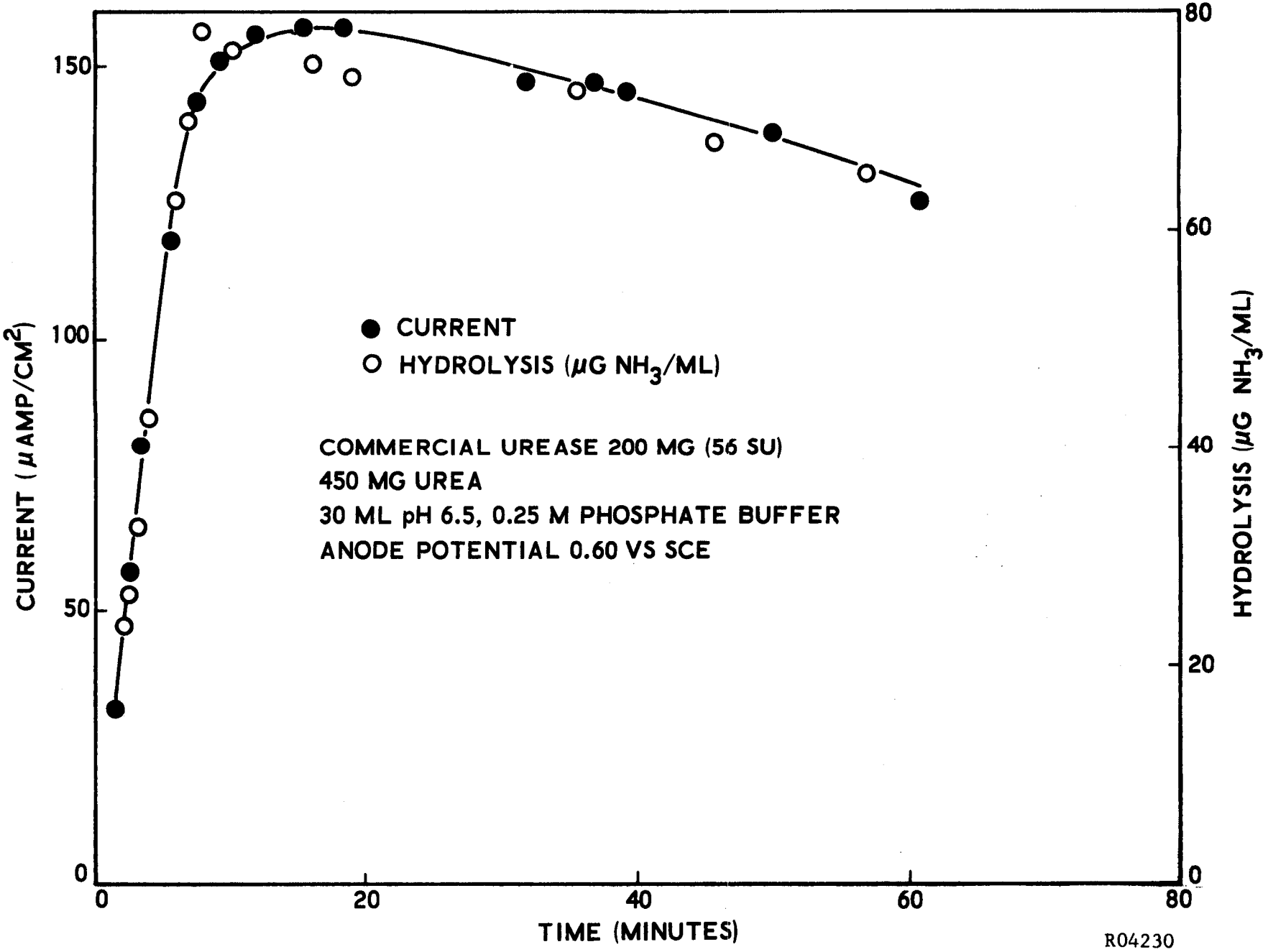

FIGURE 10. RELATION OF CELL CURRENT TO EXTENT OF UREA HYDROLYSIS

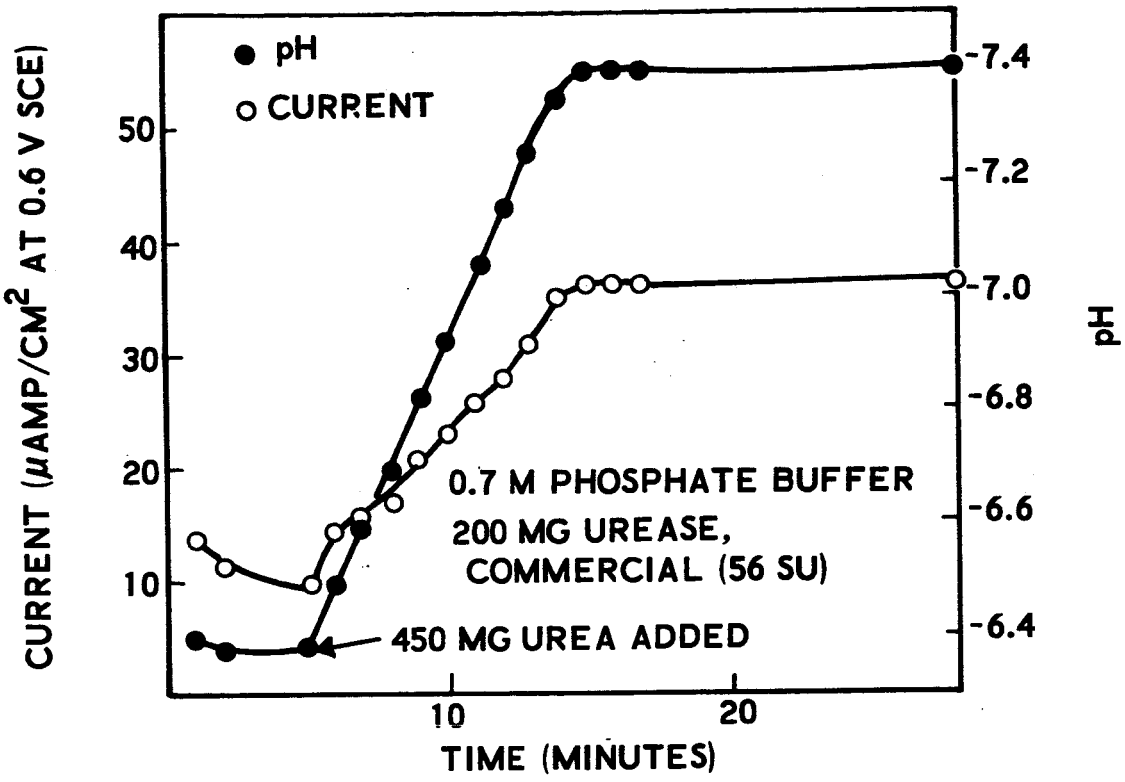

FIGURE 11. CHANGE IN CURRENT AND pH DURING UREA HYDROLYSIS

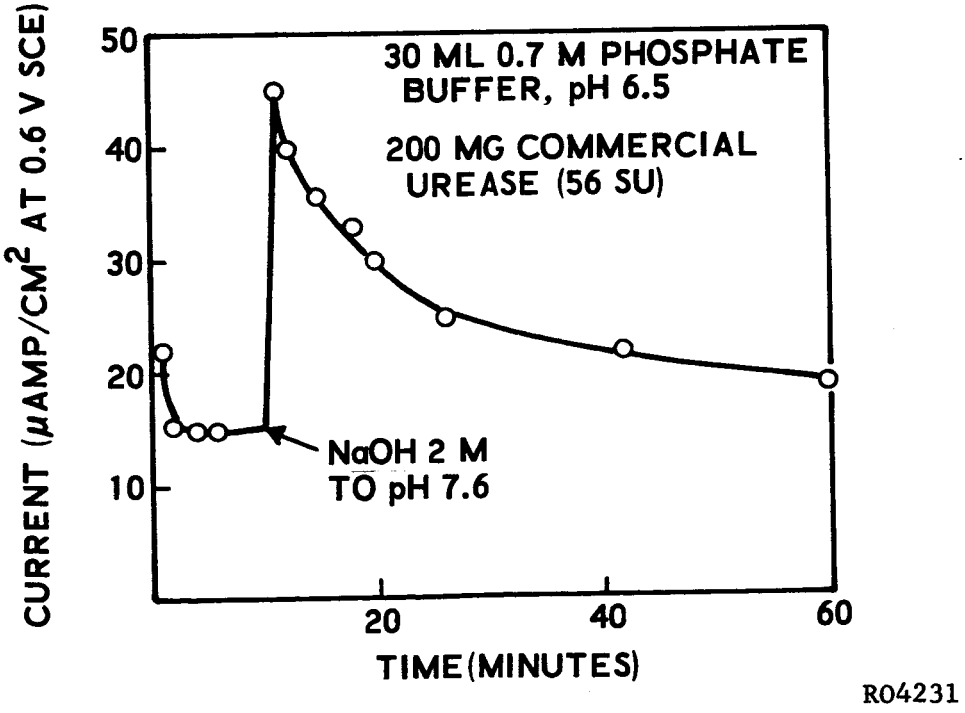

FIGURE 12. EFFECT OF pH ON UREASE CURRENT

a. Sugar Oxidases

Glucose oxidases of varying specificities may be obtained from a variety of sources. In general the reaction they perform is

$$R - \underset{\underset{CR}{|}}{\overset{\overset{H}{|}}{C}} - OH + En \longrightarrow R - \underset{\underset{OR}{|}}{C} = O + EnH_2;\ EnH_2 + O_2 \longrightarrow En + H_2O_2.$$

I II

In this reaction I and II correspond to the hemi-acetal form of the sugar and the delta-lactone of the aldonic acid, respectively. The enzymes are normally flavoproteins and might have some chance of being re-oxidized anodically in an anaerobic system. Other mediators may substitute for the oxygen, forming the oxidized mediator rather than hydrogen peroxide.

Commercial glucose oxidase is normally an enzyme obtained from fungi, particularly Penicillium or Aspergillus species. This oxidase has been studied extensively here and in other laboratories and found incapable of direct reaction although significant currents may be obtained when a mediator such as ferricyanide or 2,6-dichlorophenol-indophenol is used. Sugar oxidases from Iridophycus flaccidum (a red alga) and from oranges have different electron acceptor specificities from the fungal enzymes so these were also tested to determine relative electrochemical effects. Results were negative with these enzymes insofar as direct reactions were concerned although electron carrier mediated currents were produced. Aerobically, small currents were found in all cases as a result of the hydrogen peroxide formed in the reaction.

b. Lactic Dehydrogenase and Alcohol Dehydrogenase

Lactic dehydrogenase functions to transfer hydrogen and electrons from lactic acid to NAD with the formation of pyruvic acid. Alcohol dehydrogenase performs a similar function with alcohols, forming the corresponding aldehydes. Some opportunity might exist for direct enzyme reaction with the electrode during the transfer stage, but the mechanism usually considered for these enzymes involves a reaction occurring with both the co-enzyme and the substrate attached to the enzyme simultaneously in close juxtaposition so that any intermediate state would be of fleeting duration. In the absence of the co-enzyme the substrate-enzyme complex may form but no electron transfer is effected. Tests of these enzymes either with substrate alone or with substrate and NAD demonstrated a total lack of transfer of electrons to the anode in either case. Thus, it appears that neither the enzyme-substrate complex nor the reduced NAD is capable of reaction at a bright platinum electrode (inactivity of $NADH_2$ was confirmed by direct tests).

c. Lactic Dehydrogenase-diaphorase-NAD Coupled Reaction

Diaphorase is a flavoprotein which oxidizes $NADH_2$ aerobically or with various mediators. As indicated above, $NADH_2$ is not oxidized electrochemically

but it is conceivable that the flavoprotein which does oxidize it might react. To test this system lactic dehydrogenase was used as a reducing agent (with lactic acid) to maintain a continuous supply of the reduced NAD which, in turn, would maintain the reduced form of diaphorase. Again, little or no activity was found except when electron transfer mediators were introduced.

d. Mitochondria

Mitochondria contain a series of oxidative enzymes concerned with transferring electrons in controlled manner in such a way that each change in energy during the transfer from the substrate to the final acceptor (usually oxygen) may be efficiently stored as chemical energy in the form of ATP. From 2 to 3 ATP molecules are formed from ADP for each substrate oxidation and two electron transfer. Thus there is opportunity at a number of points for reaction in an electrochemical system. However, tests of mitochondria here revealed, as with the other oxidative systems tested, that only with an electron transfer mediator such as ferricyanide was it possible to obtain useful electrochemical energy. With such mediators currents available during oxidation of succinate were directly proportional to the concentration of the electron transfer agent as demonstrated in Figure 13. It is apparent that in this reaction, with sufficient mitochondria to utilize about 1.5 μmoles of oxygen per minute, the carrier is the limiting factor in the electrochemical reaction.

e. D-Amino Acid Oxidase

The reaction performed by DAO is rather complex. The sequence of reactions during normal aerobic oxidation is:

(1) $RCH\ NH_2COOH + En \longrightarrow RC(=NH)COOH \cdot EnH_2$

enzyme-substrate complex

(2)
- anaerobic + A $\longrightarrow RC(=NH)COOH + H_2A + En$
- aerobic O_2 $\longrightarrow RC(=NH)COOH + En + H_2O_2$

(3) $RC(=NH)COOH + H_2O \longrightarrow R\text{-}CO\text{-}COOH + NH_3$

Thus, in the normal reaction, three products are formed from the original substrate; the pyruvic acid derivative, ammonia and hydrogen peroxide. Anaerobically, it would be expected that only ammonia and reduced enzyme would be formed.

Initial tests with commercial DAO preparations were inconclusive due to the low activity of such preparations. Accordingly, higher activity samples were prepared in the Philco Laboratories. Although currents obtained with these preparations when acting on the normal test substrate, D-alanine, were low they were

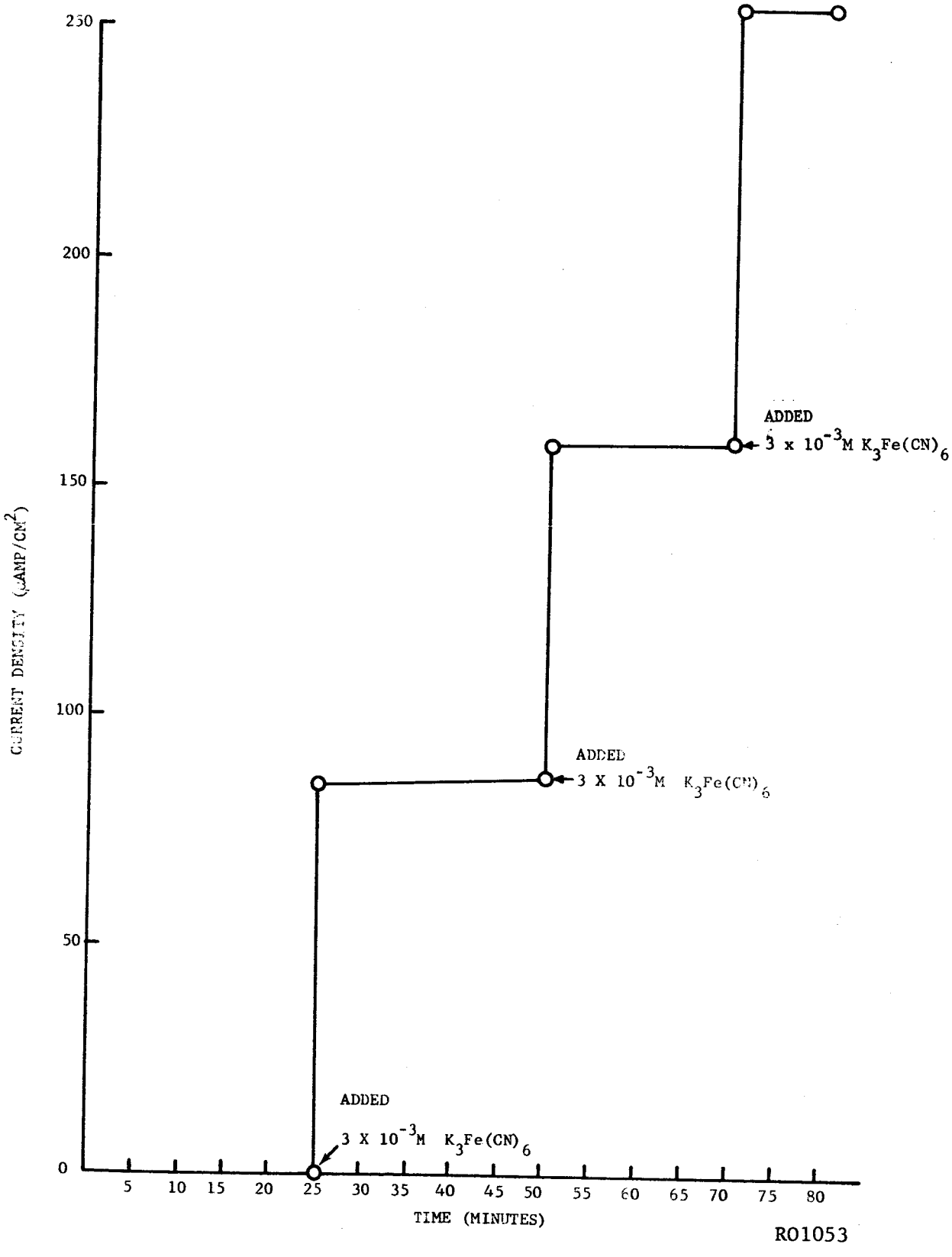

FIGURE 13. EFFECT OF $K_3Fe(CN)_6$ UPON ELECTROCHEMICAL ACTIVITY OF MITOCHRONIDA

significantly higher than obtained with other oxidative systems tested. A test of a number of additional substrates for DAO revealed that there were large differences in the ability to support a current. As shown in Figure 14, tryptophan was much superior to other amino acids. Tyrosine and histidine also had significantly higher apparent activity than alanine or serine and output was improved by use of the electron transfer agent ferricyanide.

The results shown here were obtained with cell operation procedures which apparently allowed a small amount of oxygen to enter the cell at times. Subsequent experiments with a change in procedure to obtain better anaerobic conditions resulted in complete loss of most of the apparent activity for all the amino acids. As a consequence of this discovery, trials were made in which the cell was maintained fully aerobic. Representative results are shown in Figure 15 and in 16, where the aerobic current is shown to be dependent upon the concentration of substrate, tryptophan, at constant enzyme concentration. The limiting current was also found to be dependent upon the enzyme concentration. This is shown in Figure 17 where the data is plotted as the reciprocal of the enzyme concentration against the reciprocal of the current. The meaning of the straight line curves found with this type of representation is not entirely clear at this time but it is probably related to the keto-enol equilibrium discussed later.

It becomes obvious, in the light of these data, that the major electrochemical reaction with DAO is not the reaction of the reduced enzyme with the electrode. If the reduced enzyme were reacting, oxygen would be expected to compete successfully with the anode for oxidation of the enzyme and admitting air would reduce the available current. Instead, oxygen stimulates the reaction and it is apparent that one of the products formed under aerobic conditions must be electroactive. The current yield from the enzyme reaction is still highly dependent upon the substrate used as shown in Figure 15 where the effects of aerobic conditions on utilization of tryptophan, phenylalanine and alanine are explored. Tyrosine has a limited solubility which prevents its comparison with these amino acids on the same basis as is illustrated here, but it was found that equal concentrations of tryptophan and tyrosine were equally effective. Thus, the electrochemical reaction seems dependent upon the main organic structure and therefore it would not be expected that products such as hydrogen peroxide or ammonia, products common to all reactions, would be involved in the main current production. Hydrogen peroxide was found to be responsible, however, for the small current obtained from alanine and phenylalanine. When catalase was added to the reaction medium to decompose hydrogen peroxide as rapidly as it formed, the current from tryptophan was not affected but currents failed to develop with alanine or phenylalanine, when catalase was added before admitting air, or decayed to zero, when the addition was made after the aerobic current was developed. Tests for currents with pure hydrogen peroxide at the same pH used here (pH 8.3) demonstrated that the amounts of hydrogen peroxide which might be expected as a product of these reactions would be capable of supporting the currents found in the latter two cases.

Ammonia had been found inactive under the reaction conditions used in the urease investigation and it was similarly found totally inactive under the conditions used in the DAO reaction. Therefore, it appeared that the organic product of the reaction, the pyruvic acid derivative must be responsible for the current development.

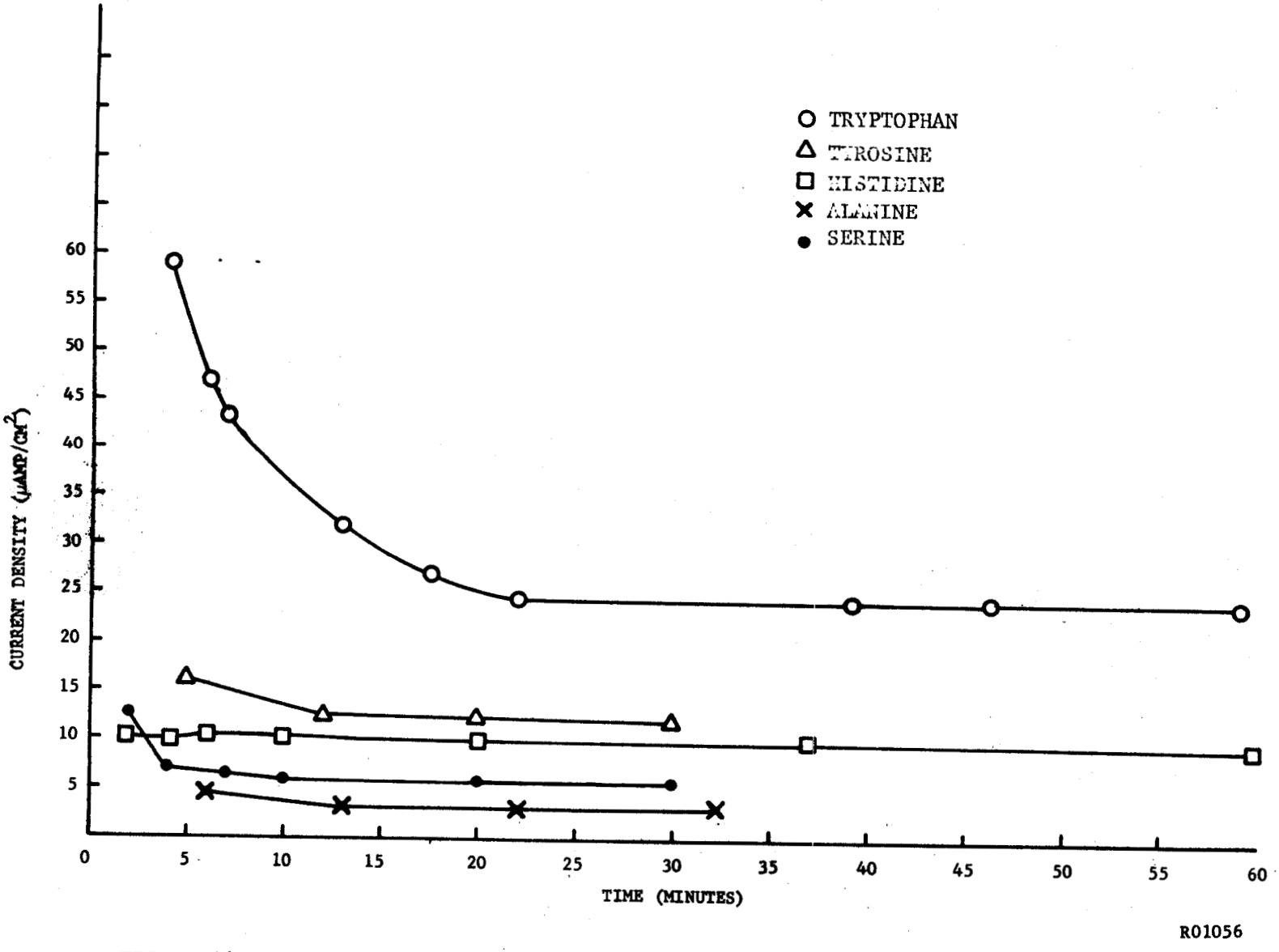

FIGURE 14. ELECTROCHEMICAL ACTIVITY OF D-AMINO ACID OXIDASE WITH DIFFERENT SUBSTRATES

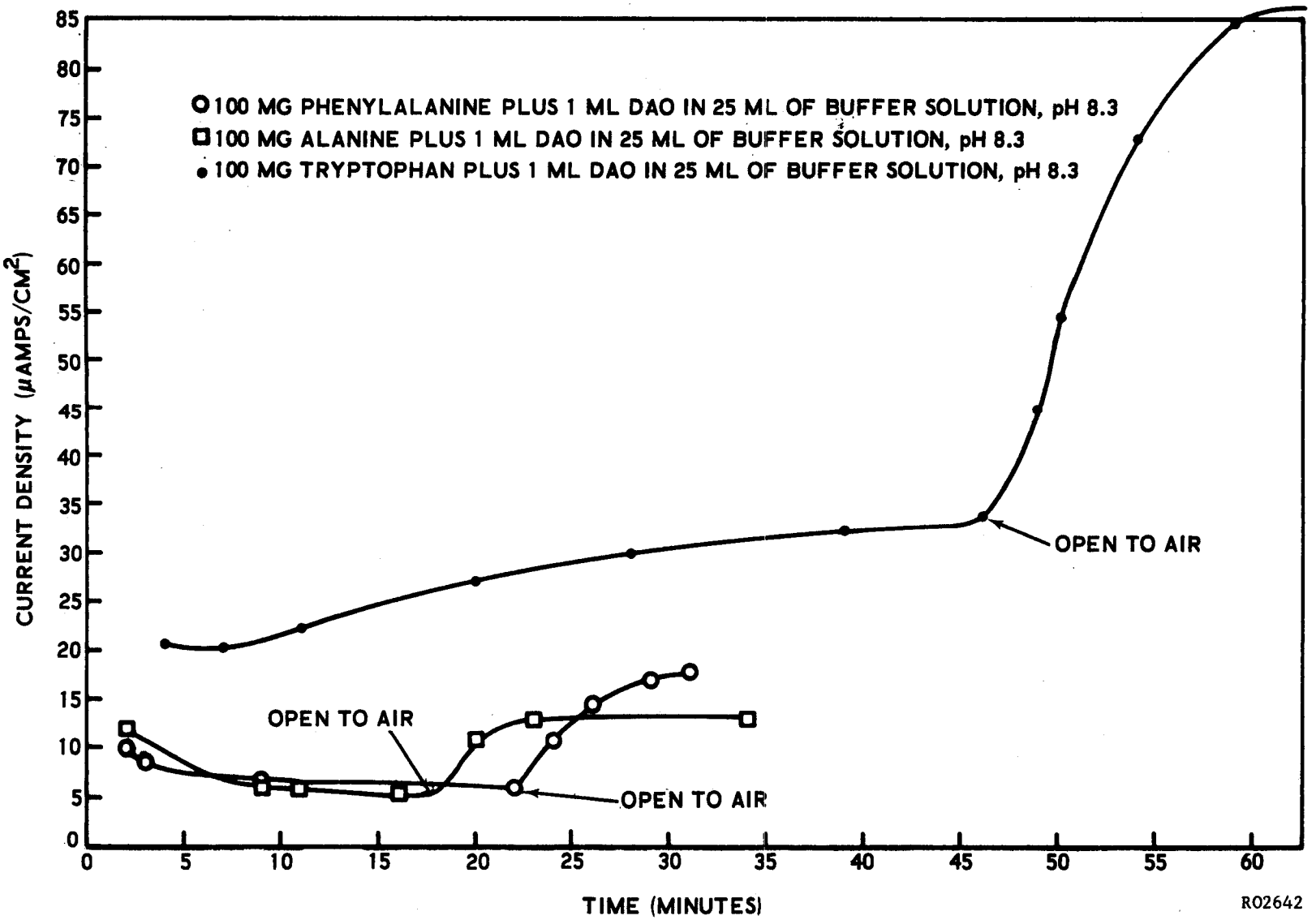

FIGURE 15. ELECTROCHEMICAL ACTIVITY OF DAO WITH DIFFERENT SUBSTRATES

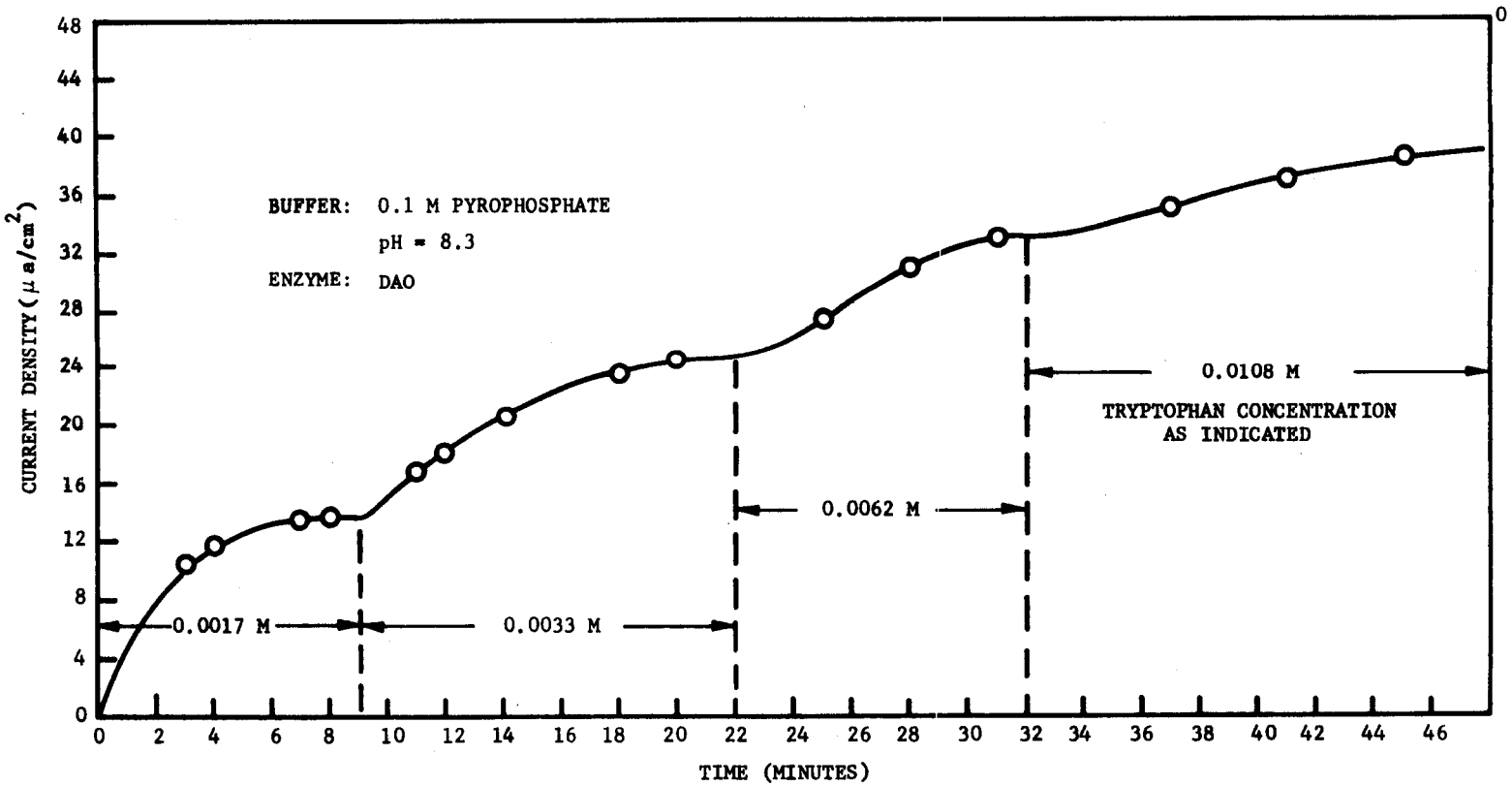

FIGURE 16. DEPENDENCE OF CURRENT UPON TRYPTOPHAN CONCENTRATION AT CONSTANT ENZYME CONCENTRATION

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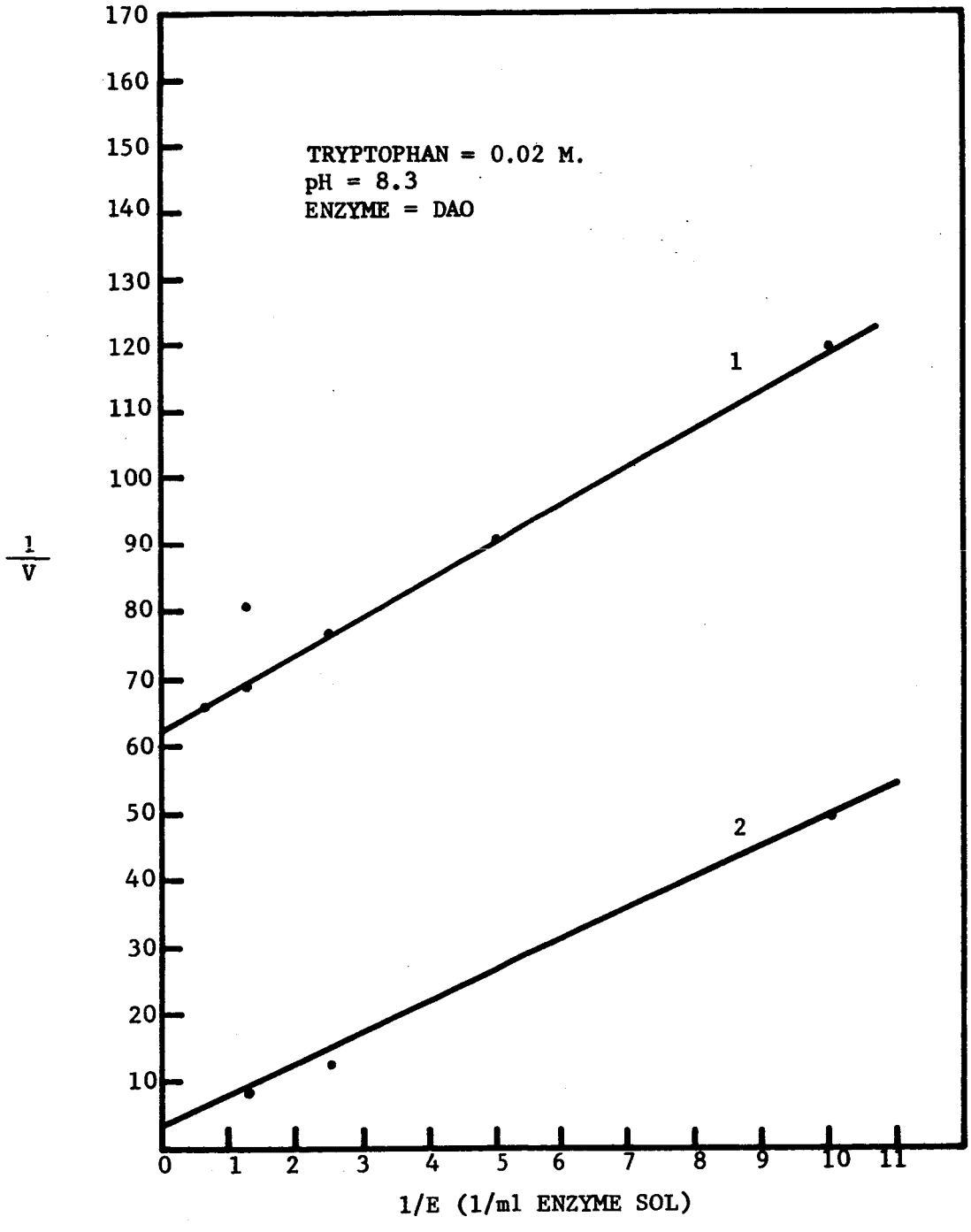


FIGURE 17. RECIPROCAL PLOTS OF ENZYME CONCENTRATIONS AND CURRENTS AT CONSTANT TRYPTOPHAN CONCENTRATION

Tests of the electrochemical characteristics of various pyruvate derivatives were started and attempts were made to establish the relation of current density to product concentration and to determine the yield and mechanism of the electrochemical reaction. This work is presented in Section 4. Tests of DAO at high concentration in attempts to detect any possible direct reaction of enzyme electrochemically are also included in Section 4. Use of DAO and tryptophan in attachment studies is given in Section 5.

3.4 REDUCTIVE ENZYMES

Normally, it might not be expected that the reductive enzyme could be expected to have a direct contribution in an electrode reaction although it may readily reduce one substrate at the expense of another to make a product which is easily oxidized anodically. It also appeared worthwhile to consider the possibility that the intermediate enzyme-substrate systems might have some degree of reactivity electrochemically. Therefore, a study of a sulfite-nitrite reductase system was undertaken as an example of a reduction sequence which had more than average possibility for direct reaction.

a. Sulfite-Nitrite Reductase from E. Coli

The $NADPH_2$ specific sulfite-nitrite reductase of E. coli carries out a rather complex set of reactions. It may use as oxidizing substrates nitrite, hydroxylamine or sulfite with only $NADPH_2$ being active as the reducing substrate. Overall reactions are given below:

$$(1)\quad NO_2^- + 3NADPH + 2H_2O \longrightarrow NH_3 + 3NADP^+ + 4OH^-$$

$$(2)\quad HSO_3^- + 3NADPH + H_2O \longrightarrow H_2S + 3NADP^+ + 4OH^-$$

$$(3)\quad NH_2OH + NADPH \longrightarrow NH_3 + NADP^+ + OH^-$$

In the reaction with either the sulfite or nitrite, no intermediate products are released into the medium and there is no exchange of labeled nitrogen from nitrite into unlabeled hydroxylamine when the two are incubated simultaneously. Thus, it appears that the substrates nitrite or sulfite remain attached to the enzyme throughout the entire six electron exchange while being reduced to ammonia or hydrogen sulfide. In regard to making use of this enzyme in an indirect, cyclic reaction for a bioelectrode, the complete reduction sequence is undesirable since a possible intermediate, hydroxylamine, is readily oxidized electrochemically. If it were possible to interfere with the full sequence in such a way as to force release of hydroxylamine after a four electron exchange, an efficient cyclic half cell might be established based upon the anodic re-oxidation of the hydroxylamine to nitrite. Hydroxylamine is actually oxidized in relatively high yield to nitrite under conditions which might be used in a bioelectrode. The data presented in Figure 18 indicate that 3.75 electrons are given up by hydroxylamine during a fast reaction, probably to nitrite, while another 0.35 electron was obtained in the slow reaction following. This compares with the variable yields from 2.0 to 4.6 electrons for the electrochemical oxidation of hydroxylamine as determined by

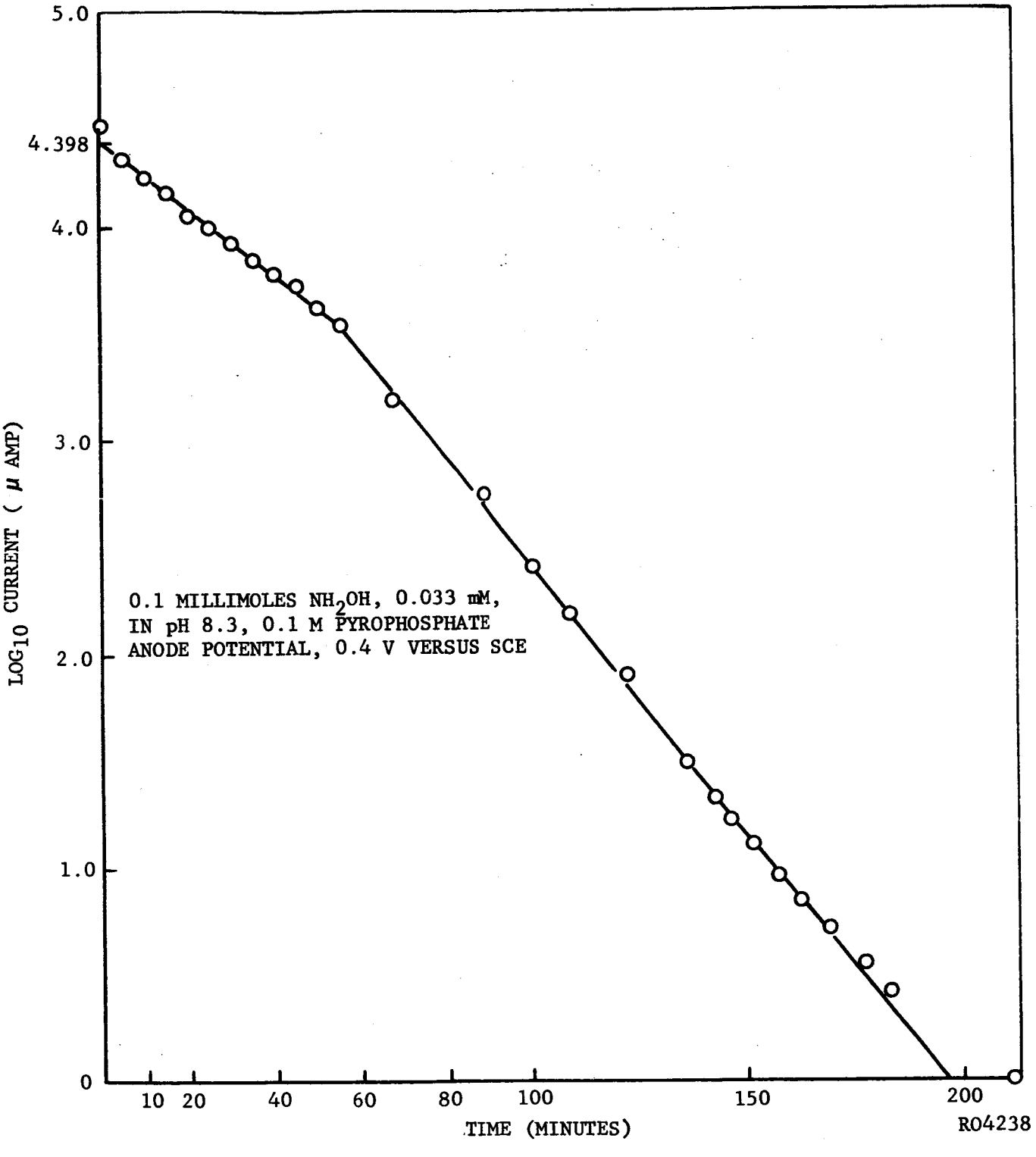


FIGURE 18. COULOMETRIC OXIDATION OF HYDROZYLAMINE

Lingane and Jones (14) or Davis (15). These authors were carrying out the oxidations in acid solution and concluded that the products were complex. In both cases, the anodic polarization potential was equal to or greater than 1.0 volt so that oxidation of nitrite to nitrate might be expected. Under the physiological conditions and low potentials used here the probable major product is nitrite. Such a reaction could be pumped by the oxidation of organic material to supply the reduced NADP required for the enzymatic formation of the hydroxylamine.

Electrochemical tests of the other substrates for the enzyme showed that neither sulfite nor nitrite (Figures 19 and 20) underwent significant oxidation under normal enzyme reaction conditions. Weak oxidation of nitrite occurred but the current found with sulfite was probably due to impurities as shown by the rapid fall of current with time.

Another possible mode of action, however, would be the reaction of the enzyme-substrate complex in the partially reduced state. Since the substrate must dwell upon the enzyme through a series of three separate reactions with $NADPH_2$, there would be a greater than normal period for other reactions to occur. Thus, it might be possible for the hydroxylamine to react electrochemically while still attached to the enzyme. This was the initial hope in testing the enzyme system. Subsequent testing of analogous models, i.e., hydroxamic acids, indicated that the amide or bound forms of hydroxylamine are not electrochemically active. The possibility exists that while such a reaction might not be expected to be highly efficient in solution, where the enzyme-substrate would have to orient itself with respect to the electrode to enable reaction, it might be efficient under some conditions with electrode attached enzymes.

Since the reaction sought was of low probability, all the tests were performed in the small cell (Figure 3). This permitted the use of enzyme molar concentrations approximating the values normally used for the substrate in the vicinity of the electrode. Enzyme was prepared, as indicated in Section 2.1 by modifications of published procedures. Substrate and buffer were placed in the chamber with the enzyme. (Enzyme concentrations were too low to enable use of the separated anolyte and electrode compartments.) Current tests were made with enzyme and substrate alone after allowing a sufficient period for reaching equilibrium. Then a sufficient amount of $NADPH_2$ was added, in a small volume of liquid, to permit almost complete reduction of the nitrite in the system. In the initial experiments, using enzyme at about 10^{-5} M the currents rose from the background current of 0.5 $\mu a/cm^2$ to about 5 $\mu a/cm^2$ upon addition of the reducing agent. Similar rise in current was not found when the enzyme was omitted in the experiments. However, in subsequent tests, with enzyme at the same or higher levels, there was little or no increase in polarizing current upon addition of the coenzyme. Thus, it was necessary to conclude that the results in the first few experiments must have been due to impurities in one of the reagents, and that, under these conditions, the reaction of the enzyme or enzyme substrate with the electrode is negligible or undetectable. It remains to be seen, however, whether such a reaction could be made efficient electrochemically through an oriented attachment or through the use of an inhibitor which might force dissociation of the substrate after only a partial reduction.

Enzymes which do not carry reduction of nitrate or nitrite to the ammonia stage, such as those of the denitrifying bacteria, might also be useful. Such enzymes, not yet adequately studied, might permit release of intermediate, electroactive products.

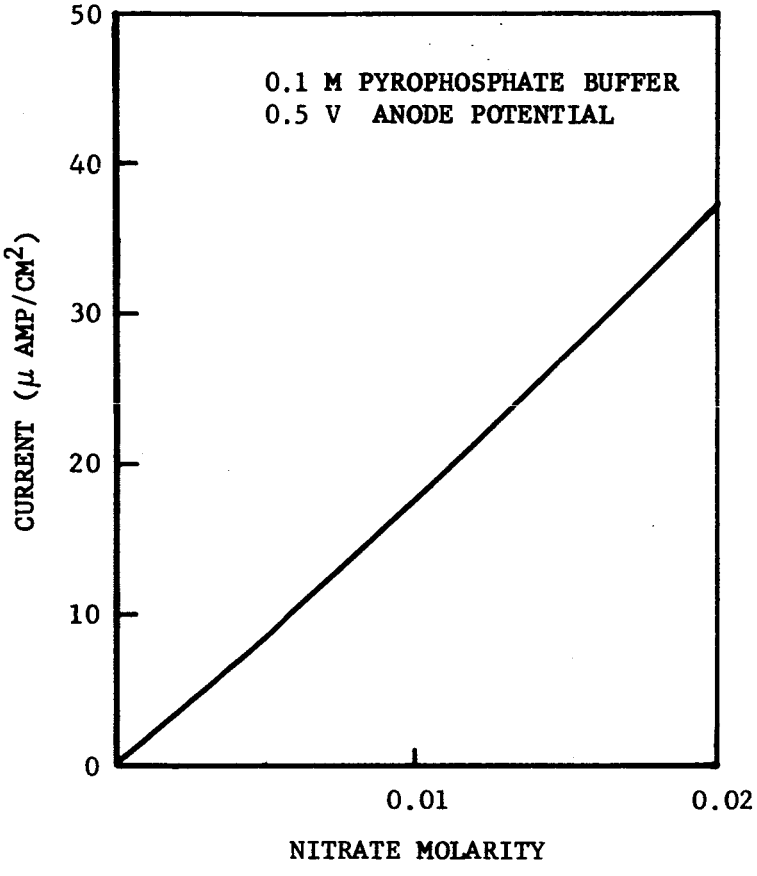


FIGURE 19. ANODIC OXIDATION OF NITRITE

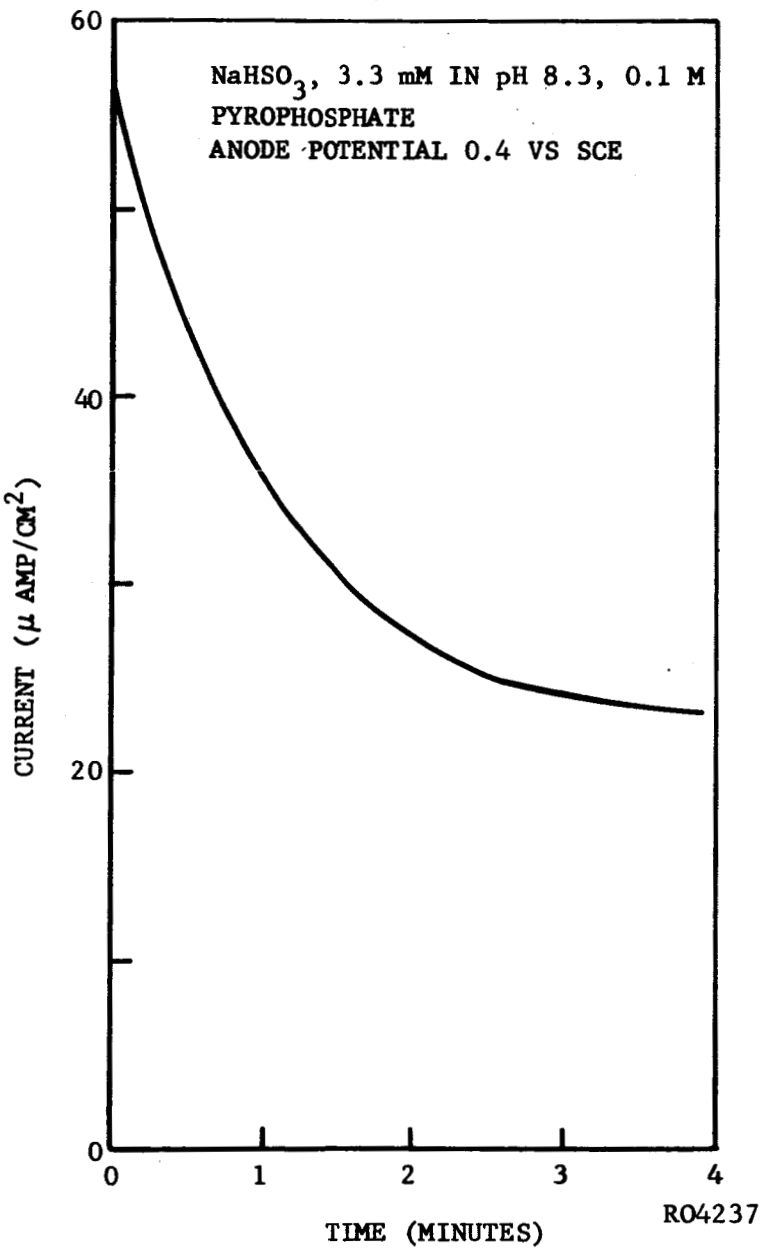


FIGURE 20. ANODIC OXIDATION OF SULFITE

SECTION 4

MECHANISM OF THE ELECTROCHEMICAL REACTION IN THE D-AMINO ACID OXIDASE SYSTEM

As indicated in the Section 3.1.2E, the D-amino acid oxidase system appeared to give rise to oxidized derivatives of the amino acid, in certain cases, which were electroactive while the amino acid was not. Other products, the ammonia and hydrogen peroxide, were excluded as possible participants in the electrochemical reaction in these cases. The production of a new electroactive species in this reaction seemed to warrant further study to characterize the reactions occurring and to attempt to determine the efficiency of the overall reaction.

4.1 ELECTROCHEMICAL REACTIVITY OF VARIOUS PYRUVIC ACID DERIVATIVES

The amino acids producing the most electrochemical activity in the DAO system were tryptophan and tyrosine (the latter on the basis of current values per unit of concentration since it is of low solubility with correspondingly low current values). Accordingly, tests were made of the electrochemical activity of the corresponding pyruvate derivatives of these amino acids, in comparison with those of amino acids having low electrochemical yield values. Figure 21 obtained with indole-3-pyruvic acid (IPA) shows a relatively high electrochemical activity with currents roughly proportional to the amount of IPA present. The first three points on this chart were obtained by adding measured aliquots of a concentrated solution of IPA to the buffer in the electrochemical cell while the points for the highest concentrations were obtained by adding solid IPA to the buffer solution and taking measurements as soon as solution was complete. As will be demonstrated later, this is the reason for the break in the curve as obtained. Table 1 shows the results obtained in similar experiments with phenylpyruvic acid (PPA) and p-hydroxyphenyl pyruvic acid (HPA). While there is no significant current obtained from the PPA, the HPA values are quite similar to those obtained for IPA at similar concentrations. Experiments with

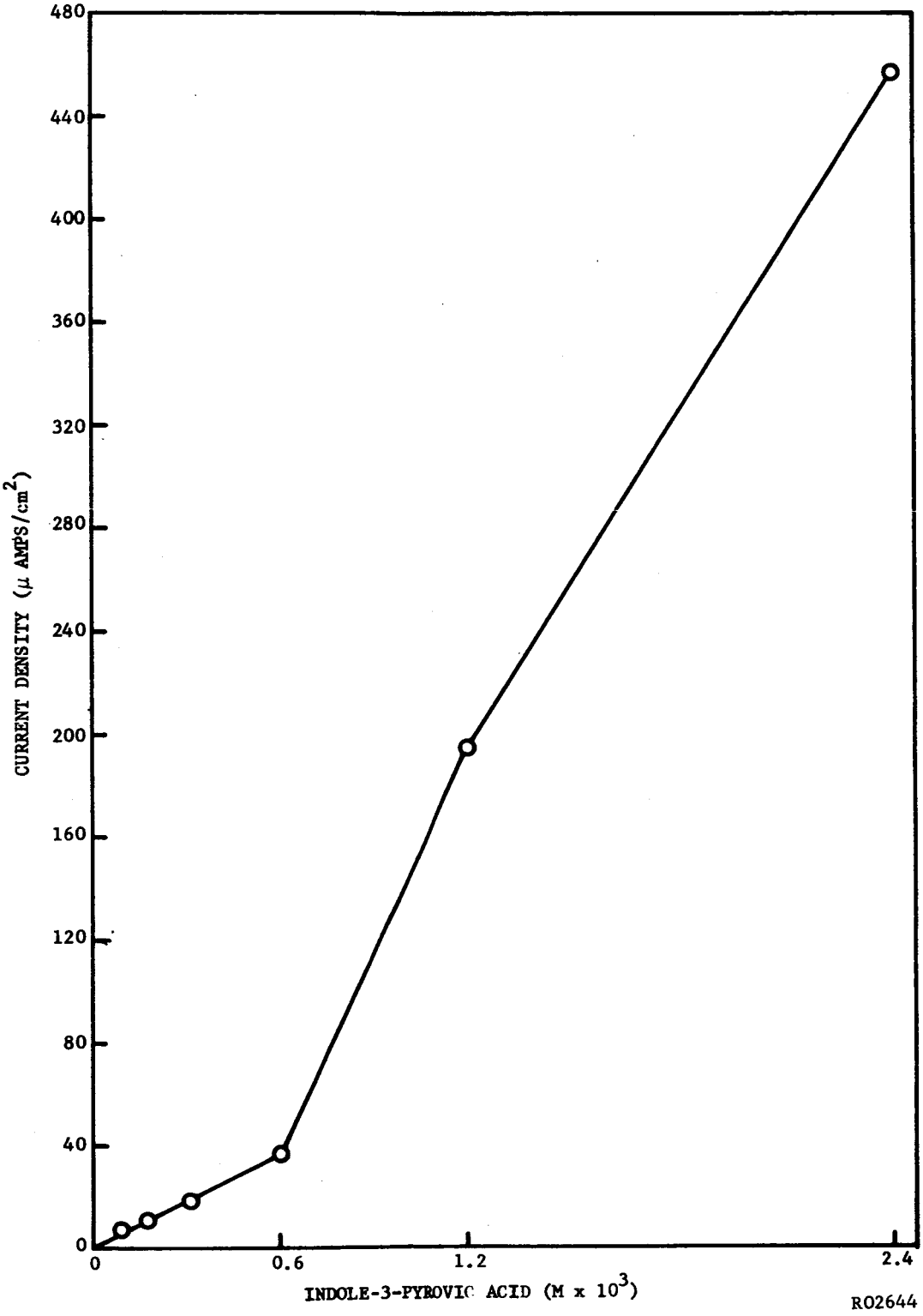


FIGURE 21. CURRENT FROM INDOLE-3 PYRUVIC ACID

TABLE 1

ELECTROCHEMICAL OXIDATION OF AROMATIC PYRUVIC ACIDS

Phenylpyruvic Acid (PPA)

Conditions: 0.1 M pyrophosphate buffer, pH 8.3, anolyte volume-25 ml, Nitrogen gas phase

Cell Contents	V_o(mv)*	V_c(mv)*	I_c(μa/cm^2)*
Buffer	+209	unstable	negligible
14.4 μg/ml PPA	+210	unstable	negligible
28.8 μg/ml PPA	+215	unstable	negligible
57.6 μg/ml PPA	+215	unstable	negligible
115 μg/ml PPA	+213	unstable	negligible

p-Hydroxyphenyl Pyruvic Acid (HPA)

Conditions: as above

Cell Contents	V_o(mv)	V_c(mv)	I_c(μa/cm^2)
Buffer	+234		
18 μg/ml HPA	+150		
35 μg/ml HPA		+200	3.5
65 μg/ml HPA		+200	5.5
114 μg/ml HPA		+200	7.9

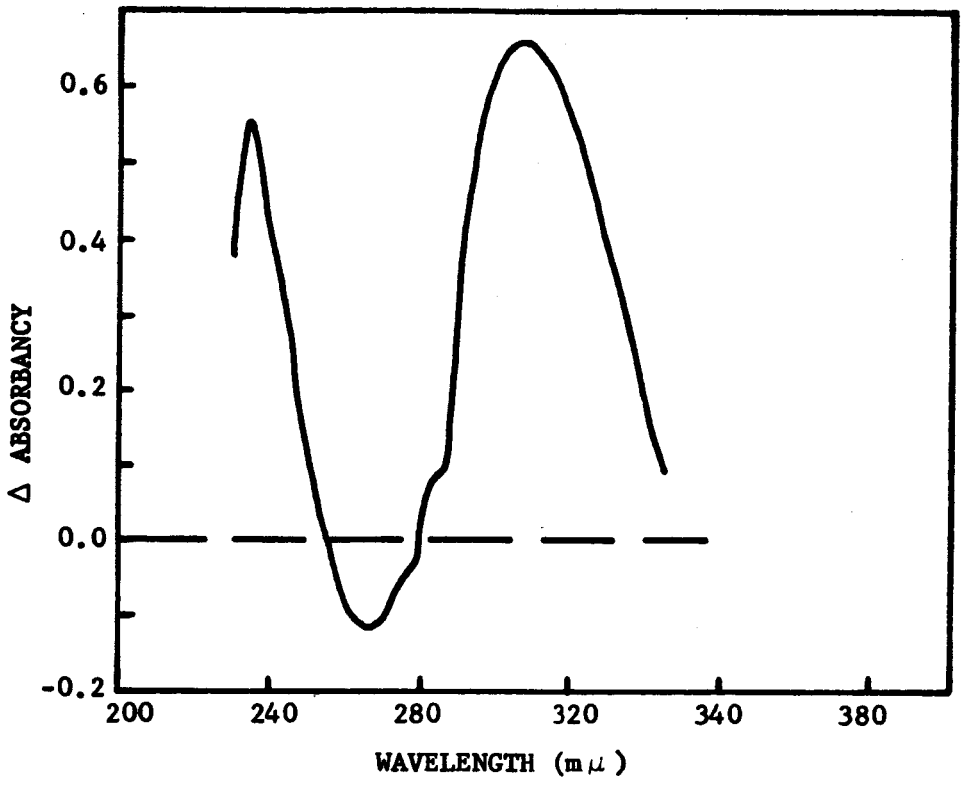
* V_o - open circuit potential
V_c - closed circuit potential
I_c - load current

pyruvic acid showed negligible electrochemical activity for the parent compound in this series. Thus, the activity appears to have a connection with the character of the aromatic ring structure and its interaction with the pyruvate side chain.

4.2 DEVELOPMENT OF ANALYTICAL PROCEDURES FOR MEASUREMENT OF IPA IN ELECTROCHEMICAL SYSTEMS

In order to measure relation of the electrochemical reaction to the formation of IPA in the enzyme system, it was essential to obtain a means of quantitative measurement of IPA in mixtures of IPA and the substrate, tryptophan. Checks of the absorption spectrum of the DAO-tryptophan reaction system revealed that there were some significant optical changes occurring during the progress of the reaction. Difference spectra for IPA-tryptophan mixtures against tryptophan reference cells showed characteristics such as those illustrated in Figure 22. Here, it may be seen that the IPA has two difference spectrum maxima, one at 306 mμ, the other at 234 mμ. There is also a small negative difference at 265 mμ. Tests were made for use of these inflection points as analytical tools by making calibration curves from varying mixtures of IPA and tryptophan against a standard tryptophan solution. Both reference and sample absorption cells contained 0.6 ml of 0.005 M D-tryptophan. A solution of 0.005 M IPA was also prepared. The latter solution was used to replace aliquots removed from the sample cell so as to obtain known concentrations of IPA in the presence of known concentrations of tryptophan. The additive total concentration of the IPA and tryptophan was always 0.005 M, corresponding to the situation which would arise during the enzymatic transformation of tryptophan in the electrochemical cell. Typical calibration curves, obtained with the use of an optical spacer to give a 1 mm light path, are given in Figure 23. Good linearity is obtained over the range investigated for the two positive maximum peaks.

A check of the accuracy of this analytical technique as applied to enzyme reactions was made by adding known quantities of IPA to the enzyme and tryptophan in the electrochemical cell and making spectrophotometric determinations of the IPA found under normal operating conditions. Under carefully anaerobic conditions, tryptophan was introduced into the electrochemical cell, either with or without enzyme (0.005 M tryptophan, 1.0 ml purified DAO). Aliquots of the cell solution were then withdrawn and replaced with similar portions of 0.005 M IPA. The syringes and solutions of IPA were preflushed with nitrogen to ensure completely anaerobic transfer and maintenance of the anaerobic conditions of the electrochemical cell. With such conditions, the IPA found should be almost entirely due to the added IPA since, without oxygen or other oxidant, the enzyme should not be able to form significant quantities of IPA from the substrate. Catalase was included in the reaction mixture to ensure that no side reactions might occur from hydrogen peroxide. For the determinations, 0.6 ml of the cell solution was transferred to a cuvette, via syringe, and scanned in the ultraviolet region using a 1 mm light path.



REFERENCE: 0.005M TRYPTOPHAN

SAMPLE: 0.0025M IPA IN 0.0025M TRYPTOPHAN

FIGURE 22. DIFFERENCE SPECTRUM FOR IPA - TRYPTOPHAN

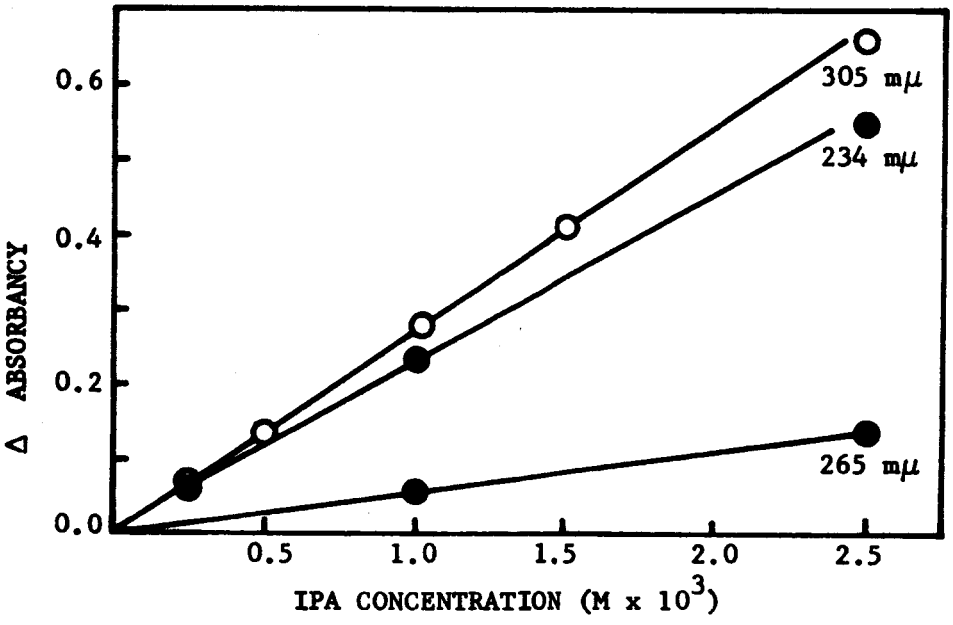


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FIGURE 23. CALIBRATION CURVES FOR IPA CONCENTRATION IN PRESENCE OF TRYPTOPHAN

Differences between the added and found IPA, based on the calibration curve of Figure 23, are given in Table 2. Determinations in the region of 0.0002 M, or higher, seem reasonably reliable with the present method of determination and some estimates may be made for lower concentrations.

TABLE 2

COMPARISON OF ADDED IPA AND FOUND IPA
DETERMINED BY SPECTROPHOTOMETRIC ANALYSIS

IPA Concentrations ($M \times 10^3$)				
Calculated From Added IPA	Spectrophotometrically Determined		Current Densities ($\mu A/cm^2$)	
	+ Enzyme	Minus Enzyme	+ Enzyme	Minus Enzyme
0	0.02	0.01	3.7	-
0.25	0.25	0.20	13	10
0.84	0.76	0.70	30	21

It should be noted that the current values found for the IPA without enzyme was less than that found for the same amount of added IPA when enzyme was present. Whether this was due to a direct effect of the enzyme on the interaction of IPA with the electrode or due to some impurity in the enzyme which affected or was affected by the IPA to produce higher currents cannot be determined at this time.

Application of the analytical procedure to an actual aerobic electrochemical system, to determine the relation of formation of IPA to current developed is given in Figure 24. DAO and catalase were added to 0.005 M D-tryptophan at time zero. An aliquot was taken immediately for photometric analysis. Some time later, under anaerobic conditions, the circuit was closed, drawing current to polarize at a potential of 0.200 v (SCE). At this time another spectrophotometric sample was withdrawn and analyzed and samples were taken at intervals for the remainder of the experiment. There was an apparent IPA concentration of 0.0001 M at the time that the discharge was started. Opening the circuit for a short time and then reclosing caused a short current surge above the previous current even though IPA concentration had not noticeably changed. Upon opening the system to the air, current immediately rose, paralleled by a rapid rise in IPA concentration. After introducing nitrogen again, the current remained essentially constant although the apparent IPA concentration continued to rise for a short time, presumably due to residual oxygen in the system (or to change in structure of the IPA as will be discussed below).

Two factors may interfere fairly strongly in the interpretation of results obtained with this analytical procedure. During the transfer of sample from the electrochemical cell to the cuvette and into the spectrophotometer for

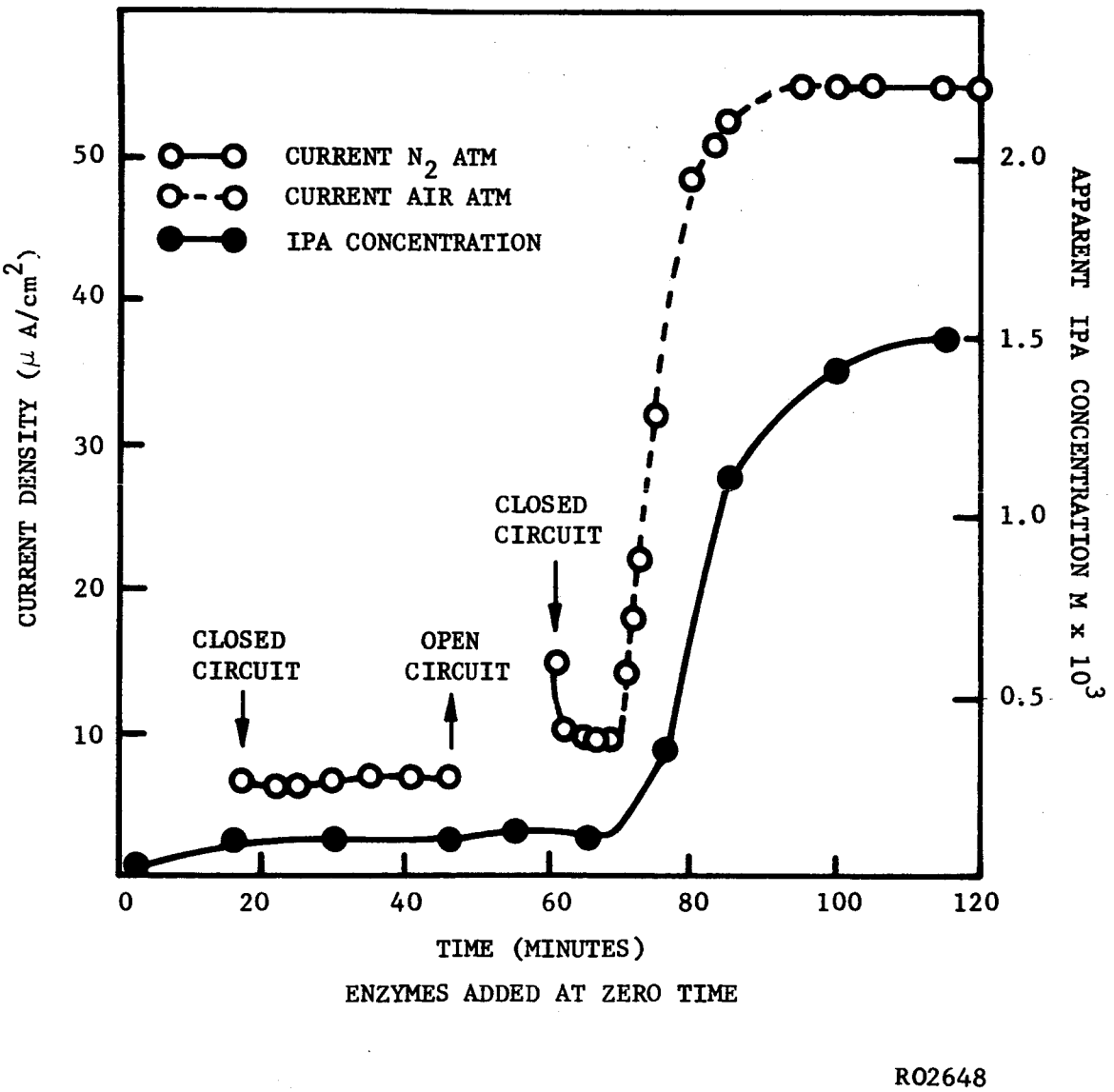

FIGURE 24. RELATION OF ENZYMATICALLY FORMED IPA TO CURRENT

measurement, there is a short period of contact with air, probably permitting a small amount of enzymatic oxidation of tryptophan. Thus, the low concentrations found during the anaerobic phase of the reaction above should probably be lower. The second factor is the change in the structure of the pyruvate, with changes in spectrophotometric characteristics, due to the keto-enol tautomerism of these compounds. Since it is not known whether the DAO reaction forms the keto or the enol form first from the amino acid, it is difficult to determine to what extent the reaction may actually have proceeded.

4.3 DETERMINATION OF THE ELECTROACTIVE FORM OF IPA AND HPA

It became evident during some of the early tests with IPA that the electrochemical activity was not always a simple function of the IPA concentration under otherwise similar conditions. Different experiments using the same solution of the IPA and buffer would not produce the same electrochemical activity, the second determination always showing lower values than the first. The problem was partially resolved on the discovery that a freshly prepared solution of IPA showed a very high electrochemical activity which decreased rather rapidly to a much lower value (see Figure 25). The current drain in these experiments was insufficient to have depleted the IPA concentration significantly so it became apparent that the IPA was changing in some manner, after solution, to produce a species which was electrochemically inactive. Spectrophotometric examination of freshly prepared solutions of IPA revealed, indeed, that extensive structural alterations must be occurring in IPA upon solution in a buffer. As illustrated in Figure 26, the freshly dissolved IPA has a very high absorption peak in the region of 320 mμ which diminishes rapidly with the appearance of lower absorbing peaks at lower wave-lengths. In the experiment illustrated, the spectra were obtained by weighing out a small quantity of IPA, dissolving in buffer under anaerobic conditions and transferring rapidly to the measuring cuvette. The total time from adding the IPA to the buffer to the start of the first scan was under two minutes. Subsequently, it was discovered that air made no difference in the rate of change of the spectrum and that it was possible to prepare concentrated solutions of IPA in methanol where the form having the high absorption peak was completely stable so that the course of change in aqueous solutions could be followed by adding small amounts of the methanol solution to the buffer already in a measuring cuvette.

Figure 27 shows the effect of varied solvents upon the spectrum found for IPA. The highly absorbing form was far more stable in organic media or acid than it was in water or buffers.

Similar tests with PPA (Figure 28) and HPA (Figure 29) demonstrated that these compounds also underwent similar changes in aqueous solution.

Examination of the change in HPA spectrum as a function of time and solvent showed that the rate of change from one form to the other was affected by pH, ionic strength and, to a lesser degree, specific ions (Figure 30). Thus, the rate of change was very slow in water alone but accelerated somewhat by the addition of

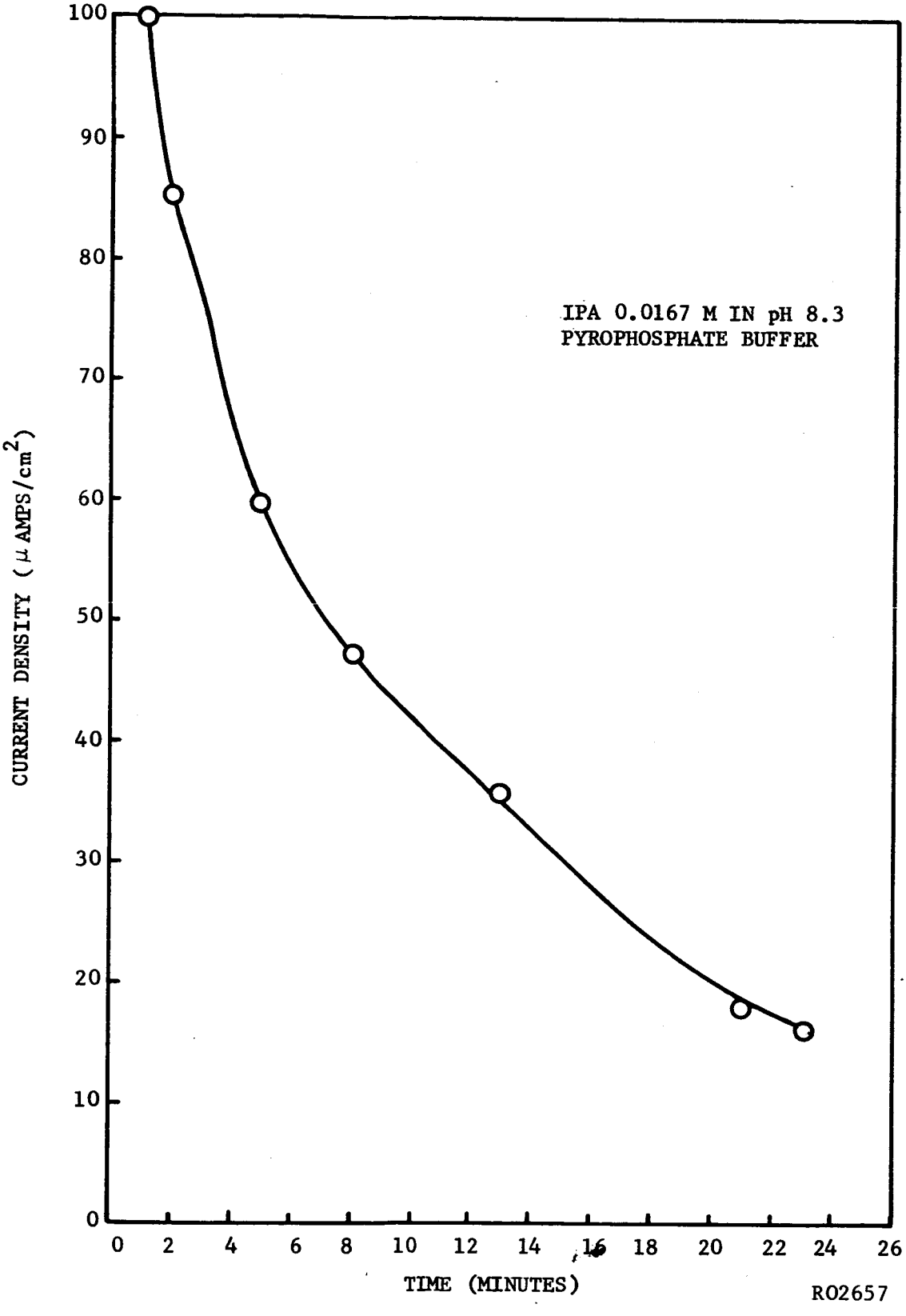

FIGURE 25. CHANGE IN CURRENT WITH TIME FOR FRESHLY DISSOLVED IPA

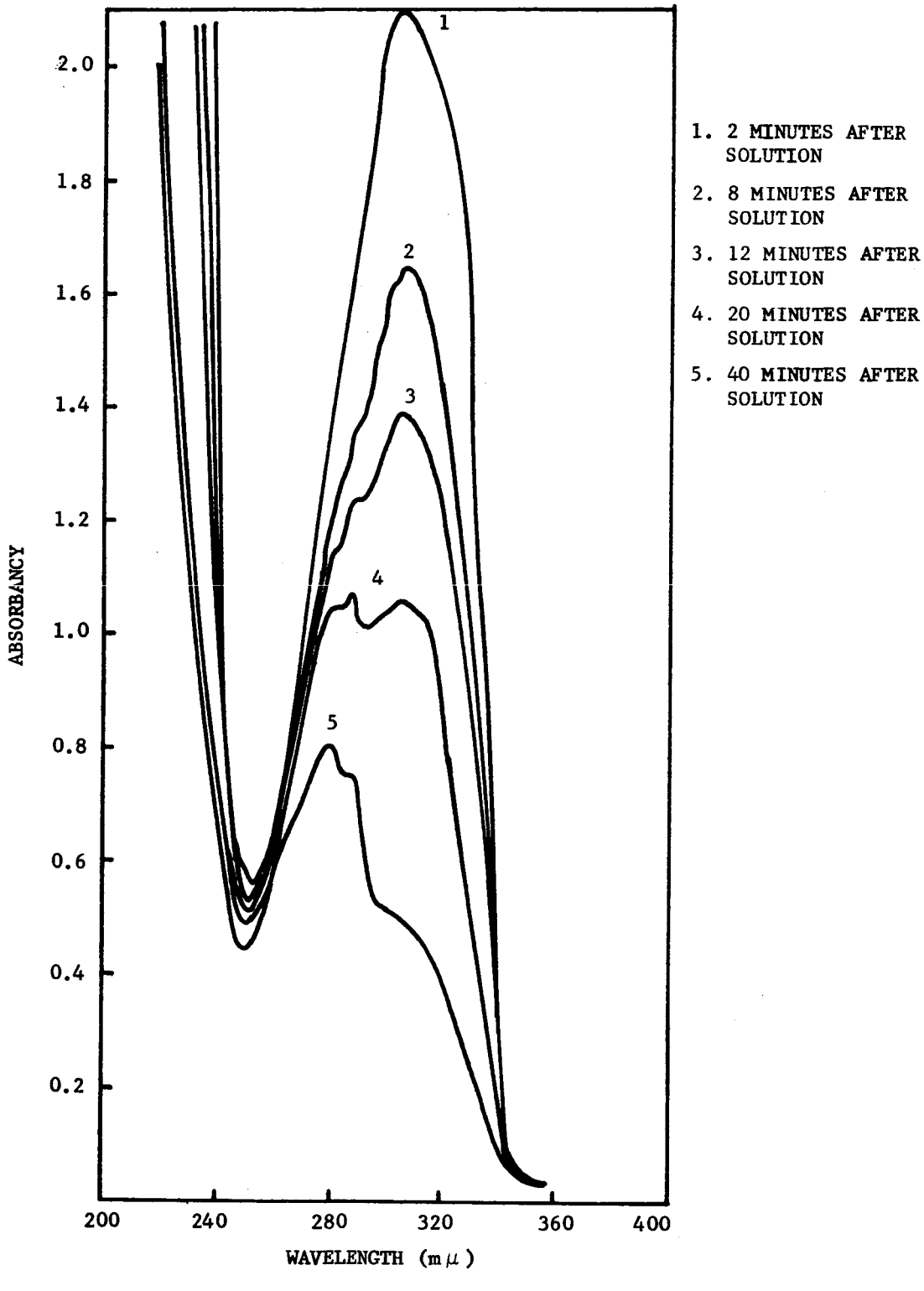

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FIGURE 26. ABSORPTION SPECTRA OF IPA (ABOUT 0.05 MG/ML) IN pH 8.3 BUFFER, ANAEROBIC

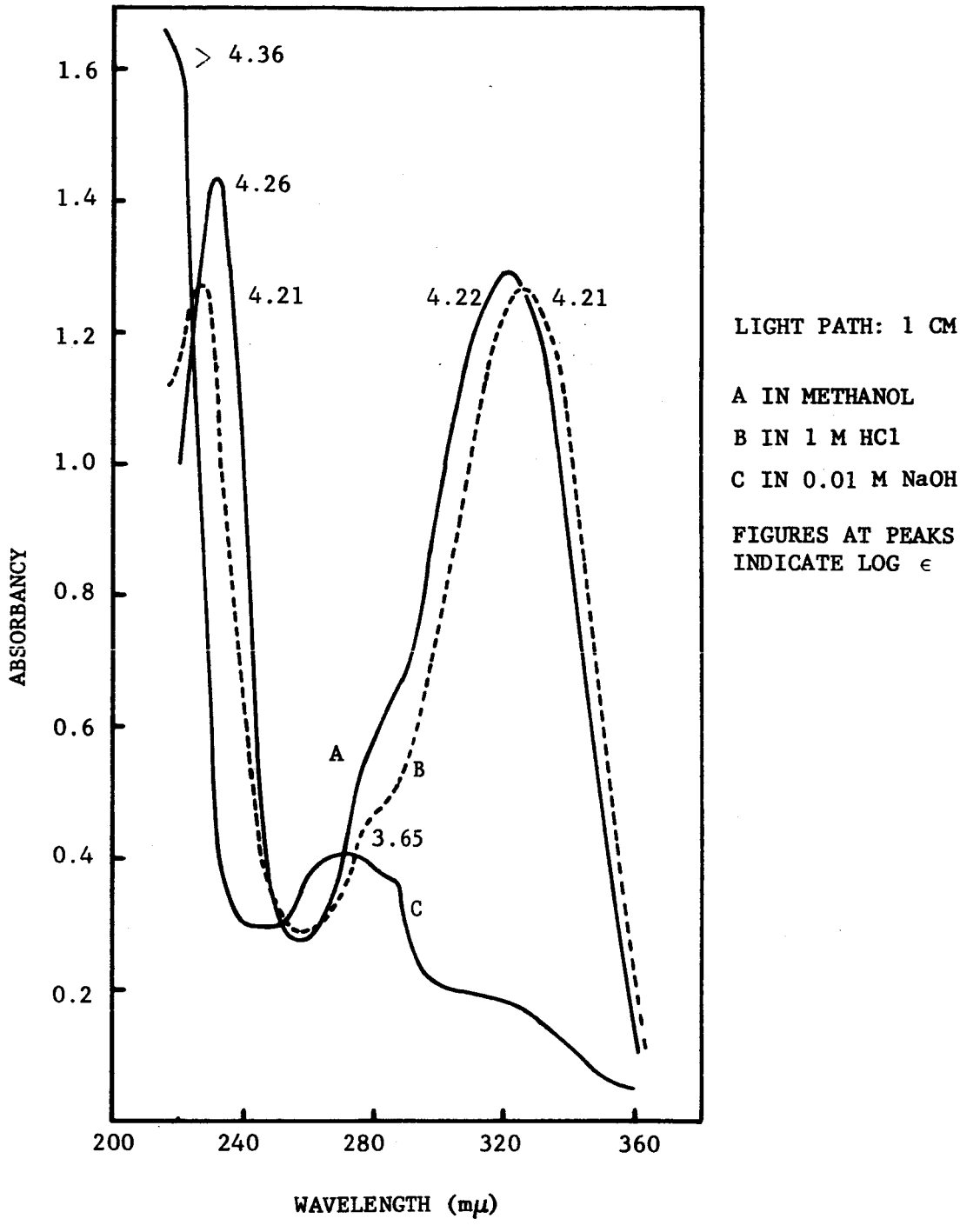

FIGURE 27. U-V SPECTRA OF INDOLE-3 PYRUVIC ACID (7.9×10^{-5} M) IN VARIOUS SOLVENTS

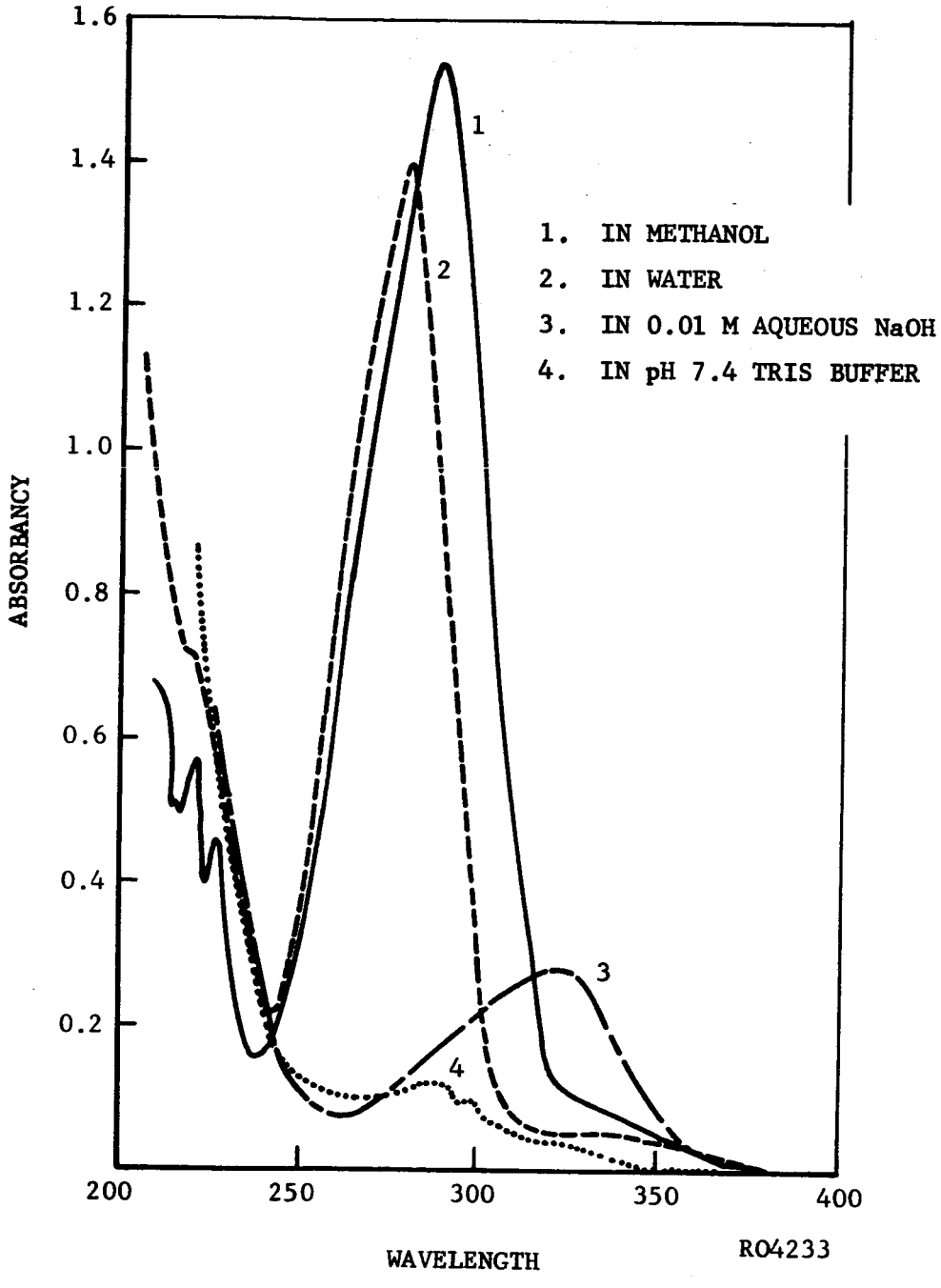

FIGURE 28. SPECTRA OF PHENYL PYRUVIC ACID IN VARIOUS SOLVENTS

A. HPA IN METHANOL, 8.66 μG/ML. NO CHANGE IN SPECTRUM WITH TIME.

B. HPA IN WATER, 8.66 μG/ML. SEE FIGURE 10 FOR CHANGE WITH TIME.

C. HPA IN, 8.56 μG/ML, IN 0.01 M SULFURIC ACID. LITTLE OR NO CHANGE WITH TIME THE CURVE FOR HPA IN METHANOLIC SULFURIC ACID WAS ALMOST IDENTICAL.

D. HPA, 8.56 μG/ML, IN AQUEOUS, 0.01 M NaOH. SLOW CHANGE WITH TIME, SHIFTING PEAK AND CHANGING INTENSITY. SIMILAR CURVE FOR METHANOLIC NaOH WITH PEAK AT 327 mμ

E. HPA, 8.6 μG/ML, in pH 8.3, 0.1 M PYROPHOSPHATE BUFFER, 20 MINUTES AFTER INITIAL SOLUTION OF HPA.

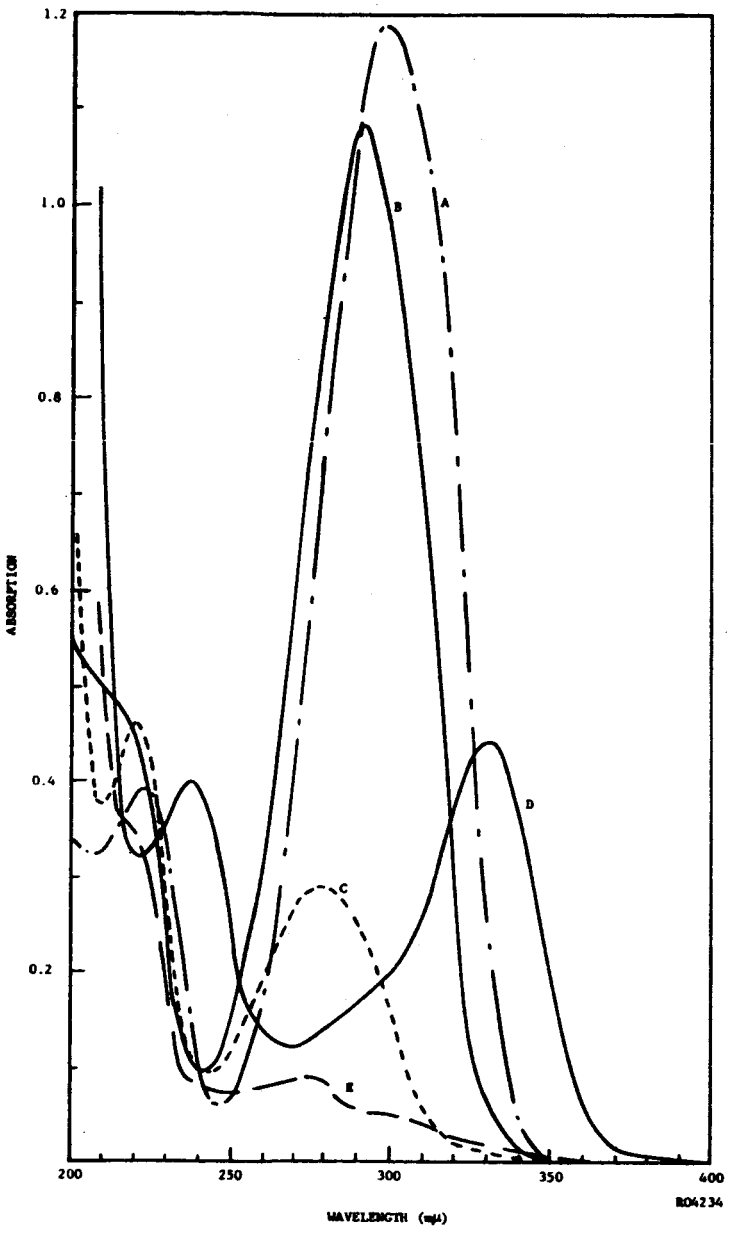

FIGURE 29. SPECTRA OF p-HYDROXYPHENYL PYRUVIC ACID

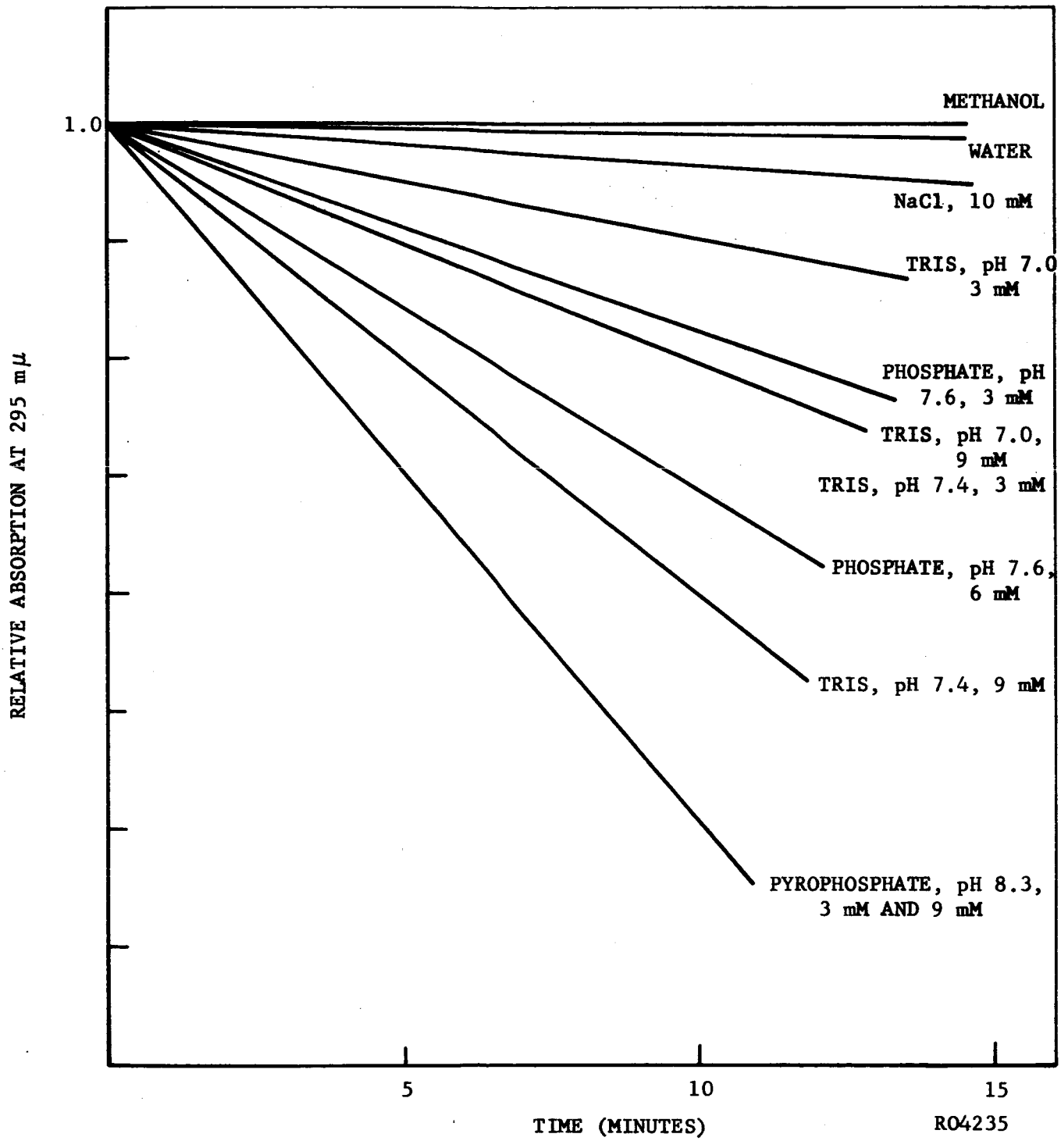

FIGURE 30. RATE OF CHANGE OF HPA ABSORPTION SPECTRUM AS AFFECTED BY IONS AND pH

NaCl. Buffer at pH 7 (Tris) was not as effective in promoting the change as the same buffer at pH 7.4. But Tris buffer at pH 7.4 was slightly more effective than phosphate buffer at pH 7.6 at the same concentration. In most instances, there is almost a direct relation between the rate of change and the concentration of the buffer but with pyrophosphate at pH 8.3 the buffer is equally effective at either 3 or 9 millimolar concentration.

Thus, all the aromatic pyruvic acid derivatives are capable of changing structure, in some manner, in making the transition from crystal to aqueous solution, and this alteration is affected by a variety of factors, high pH promoting the formation of a low absorbancy system, organic solvents or low pH favoring the high absorbancy form. It is possible to obtain the crystalline sodium salts of the aromatic pyruvates and these compounds showed the spectrum characteristic of high pH solutions immediately upon dissolving but the high absorbancy form could be readily regenerated by crystallization of the free acid, from organic solvents.

One of the obvious possibilities for a structure shift in these compounds is the keto-enol tautomerism found, to a greater or lesser degree, in any keto compound. It is well known that, in the presence of stabilizing, resonating structures, some ketone groups may exist almost entirely in the enol form. The enol form is also favored by organic solvents and low pH while the keto form is promoted by aqueous systems and high pH (except where conditions are favorable for dissociation of the enolic hydrogen as in highly basic, nonaqueous systems). All these factors are consistent with the interpretation that the IPA and HPA exist in keto and enol forms and it is the enolic form which is undergoing the electrochemical oxidation. Figure 31 indicates the change in structure to be expected for enolization of IPA. The reaction A to B is supported by many examples in the literature showing spectral shifts during the change from keto to enol form, or the reverse, similar to those observed here.

However, it is not simply the enol form which enables reaction since it is apparent, from the spectral studies, that PPA enolizes just as readily as do HPA and IPA. Thus, there is a further effect associated with the aromatic ring structure, which is the only difference between these three compounds. In the indole nucleus there is a very high concentration of pi electrons in the region of the nitrogen in the ring and similarly, in phenol the pi electron density is much greater in the region of the carbon associated with the hydroxyl group. This leads to opportunities for further resonating, structural shifts, as exemplified by C in Figure 31. Thus, it becomes quite likely that the oxidative attack in the electrochemical reaction occurs with a modified enolic form of IPA and HPA and it is possible that the oxidation occurs in the ring structure. A similar situation has apparently been found to exist in the benzophenone molecule where auto-oxidation seems to occur with the enolic form only, rather than the keto form (16). The keto-enol shift has a very real effect upon the oxidation potential of the IPA molecule, as will be shown in the chronopotentiometric investigations of the oxidation of IPA.

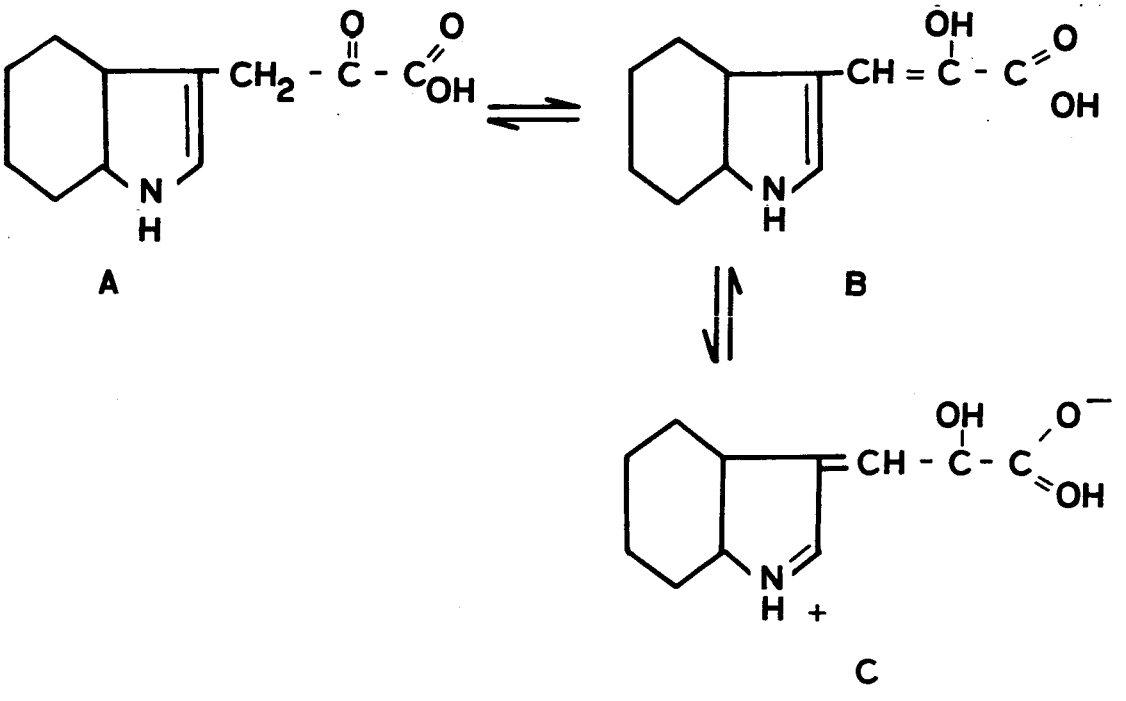

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FIGURE 31. KETO-ENOL TAUTOMERISM OF IPA

4.4 DETERMINATION OF ELECTROCHEMICAL YIELDS IN THE DAO-TRYPTOPHAN SYSTEM

A one step oxidation involving a two electron transfer in the electrochemical oxidation of IPA should give rise to 193,000 coulombs of current per mole of IPA. A test of IPA under controlled conditions in an attempt to determine the electrochemical yield, using 1.78 μmoles IPA, and oxidizing at 0.4 v for a long period of time, yielded data indicating that only a small fraction of the available IPA was being oxidized (Figure 32). In the period of time shown, less than 15,000 microcoulombs current were obtained of a possible 170,000. The final current of 2 μa remained very constant over a long period of time and it would, at this rate, require about 24 hours to complete the reaction assuming no further decrease in current as the concentration of IPA dropped. It is obvious, from the form of the curve, that a simple Nernst relation is not obeyed since the logarithmic curve does not conform to a straight line and the current is essentially constant toward the end of the reaction. It made little difference whether current was drawn at all times, or not. The current at a given time was far more dependent upon the time elapsed from dissolving than to the anodic oxidation. A solution, allowed to stand for the same period of time that the sample was being tested, would give rise to a current identical with the final current obtained for the sample when the standby solution was used to replace the original sample.

4.5 CHRONOPOTENTIOMETRIC STUDIES OF THE DAO-TRYPTOPHAN SYSTEM

The chronopotentiometric apparatus used in these studies was calibrated by the chronopotentiometric oxidation of ferrocyanide to test the reproducibility, reliability and correspondence of constants found to those determined in other laboratories. Tests proved the reliability of the system in every way.

When attention was given to the DAO system, the first material tested was tryptophan, the substrate in the reaction. It was discovered that chronopotentiometric curves varied from one determination to the next, with oxidizing potentials increasing and the duration of the potential halt decreasing in sequential determinations. Investigation of this phenomenon revealed that the chronopotentiometric oxidation of ferricyanide was also inhibited progressively in the same manner if the oxidation was carried out in the presence of tryptophan. Thus, it appeared that the electrochemical oxidation of tryptophan was causing the inactivation of the electrode in some manner so that the normal procedure of a short cathodizing process to reduce the electrode was no longer effective in preparing the surface for the next chronopotentiometric oxidation. Therefore, an altered procedure was tested for preparing the electrodes for the reaction, as outlined in Procedures, Section 2.2d. This acid, alternate cathodizing, anodizing treatment is referred to as "cleaning" and the electrode so treated a "clean" electrode. The effect of the tryptophan oxidation upon subsequent reaction and the return of the electrode to normal operation by the "cleaning" treatment are illustrated in Figure 33. It may be seen that the chronopotentiometric oxidation of tryptophan for the first time on a clean electrode gives rise to a potential halt at slightly above 0.6 v. In the subsequent determination, under identical conditions except

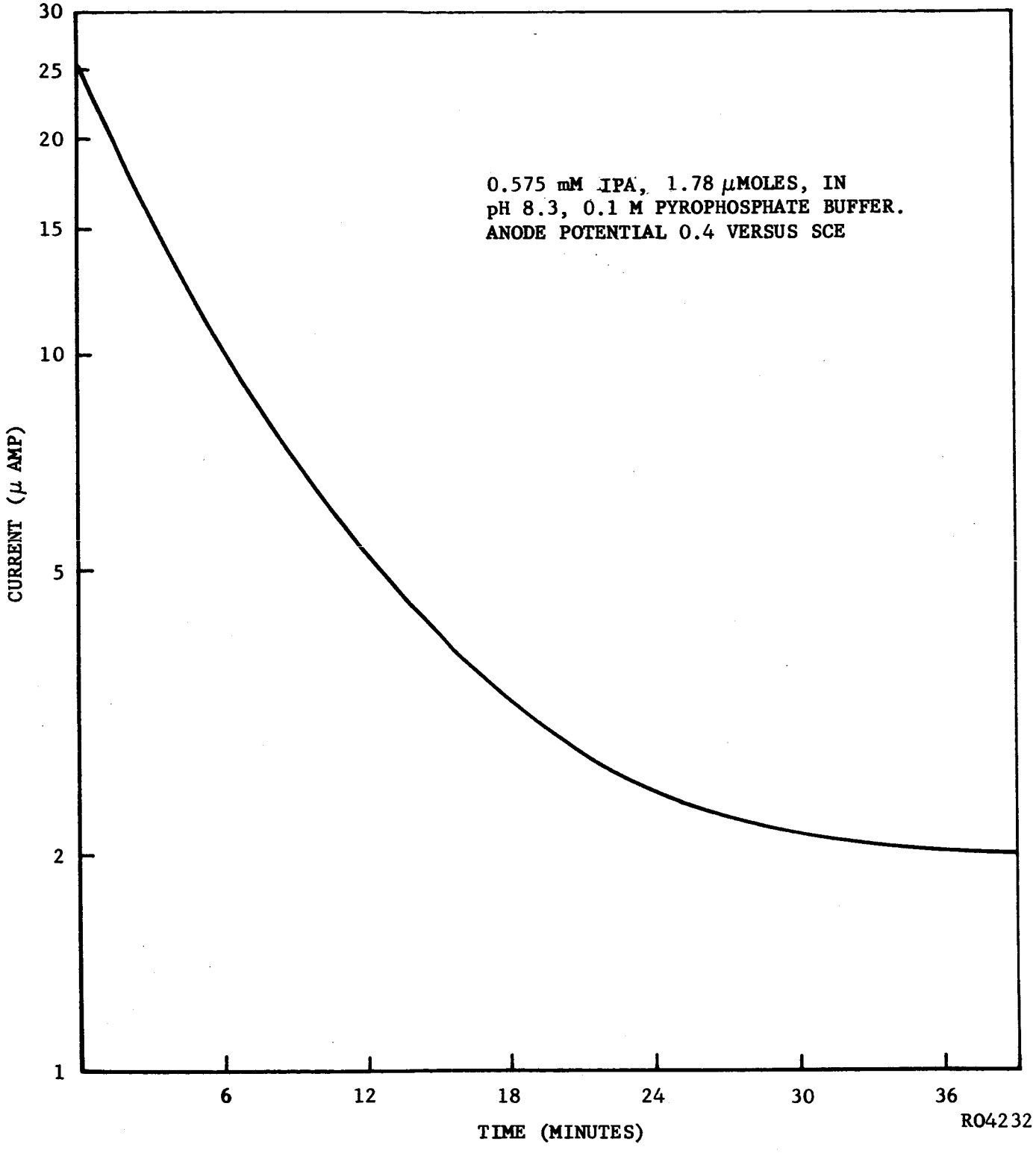

FIGURE 32. COULOMETRIC OXIDATION OF INDOLE-3-PYRUVATE

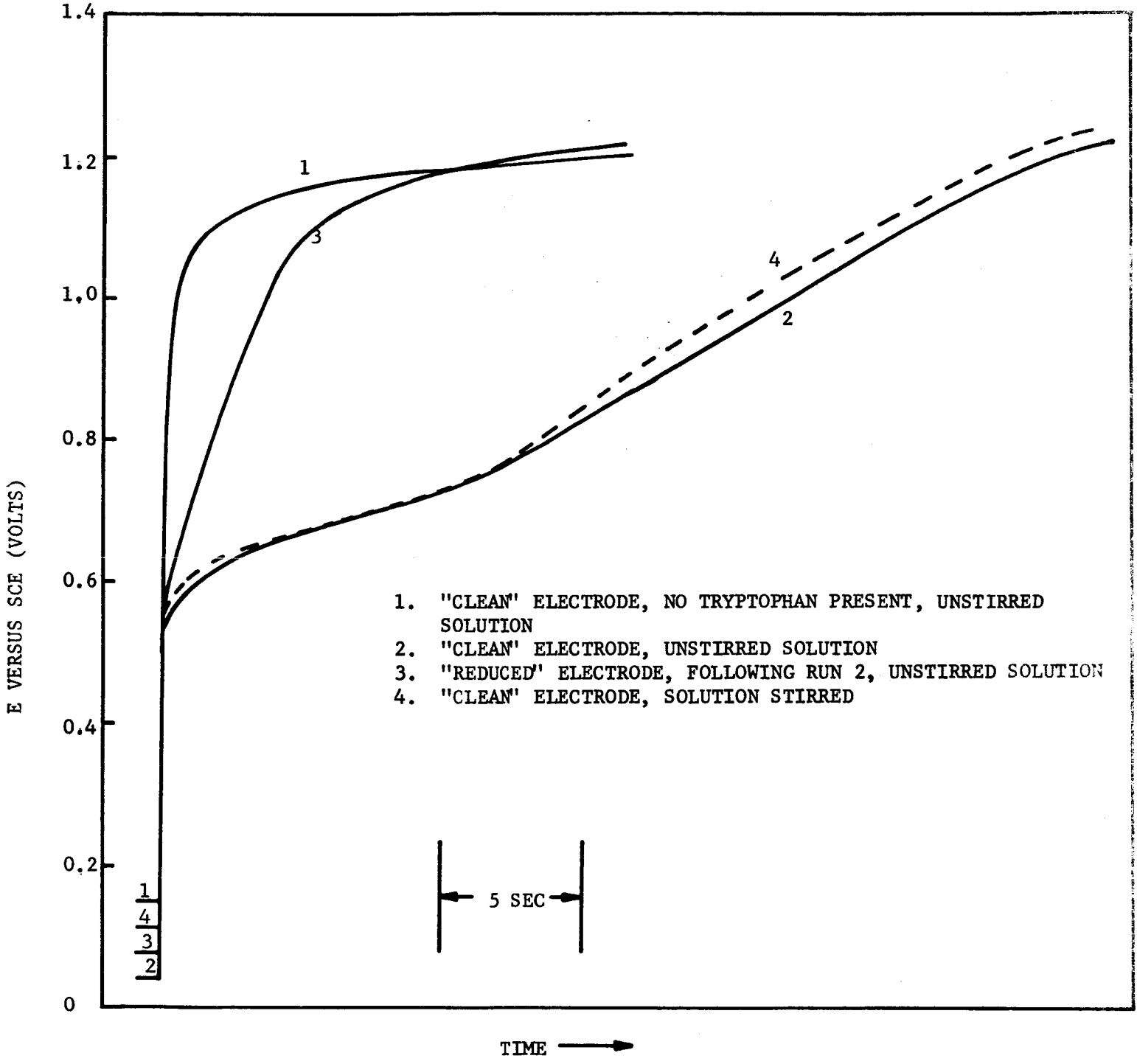

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FIGURE 33. ANODIC CHRONOPOTENTIOGRAM FOR OXIDATION OF 5×10^{-3} M D-TRYPTOPHAN IN 0.1 M $Na_4P_2O_7$ BUFFER SOLUTION, pH 8.3, i = 100 μa

for the prior run, the potential-time curve deviates only slightly from that found for buffer alone, without tryptophan present. By obtaining, once more, a "clean" electrode it is possible to reproduce curve 2 over and over again. Another item of note, however, is the fact that stirring makes no difference in the curve obtained for the chronopotentiometric oxidation of tryptophan. It appears, therefore, that an adsorptive process controls the electrochemical reaction of tryptophan rather than the diffusion process. These results are similar to those of Anson and Schultz (17) who reported that transition times for oxalic acid were independent of stirring in buffered solutions above pH 3. They were able to account for this behavior on the basis of an oxalic acid adsorption mechanism and on the fact that extensive oxidation of the platinum surface occurs at pH values above 3.

Further support for the adsorption mechanism for tryptophan oxidation was derived from the plot of $i\tau^{1/2}$ (Figure 34). According to the diagnostic criterion developed by Reinmuth (11), a positive slope on this type of plot predicts that the reaction involves an adsorption process.

The absence of the potential arrest on curve 3, Figure 33, indicates that essentially no oxidation of tryptophan occurs on the reduced electrode at this pH. Similar results were obtained on "reduced" electrodes with tryptophan in 1 N sulfuric acid. This means that the "poisoning" is due to tryptophan oxidation, rather than simply to excessive oxidation of the platinum at the high pH.

The oxidation wave of IPA at 5×10^{-3} M on a clean electrode (pyrophosphate buffer) was found to occur at $E_{\tau/4} = 0.61$ to 0.63 v (SCE). The initial experiments were carried out with solution which had been allowed to stand for some time prior to the first determination. Transition time determinations over a range of currents were determined and values of $i\tau^{1/2}$ are tabulated in Table 3. The constancy of these values indicates that the oxidation in this instance is diffusionally controlled. It was not possible from an analysis of the values of

$$\left[\frac{\tau^{1/2} - t^{1/2}}{t^{1/2}} \right]$$

versus E or

$$\log \left[1 - \frac{t^{1/2}}{\tau} \right]$$

versus E whether the oxidation process was reversible or irreversible since neither plot conformed to expected curves.

There are two other points of interest in the electrochemical oxidation of IPA. It was found that the oxidation of IPA also poisons the electrode in the same manner as found for tryptophan. This is demonstrated in Figure 35 where curve 1 shows the long potential halt characteristic of the oxidation on the "clean" electrode while curve 2 shows the much reduced potential halt obtained when the oxidation is performed at an electrode which has not had the drastic cleaning treatment. Curves 3 and 4 show the same comparisons using a different current.

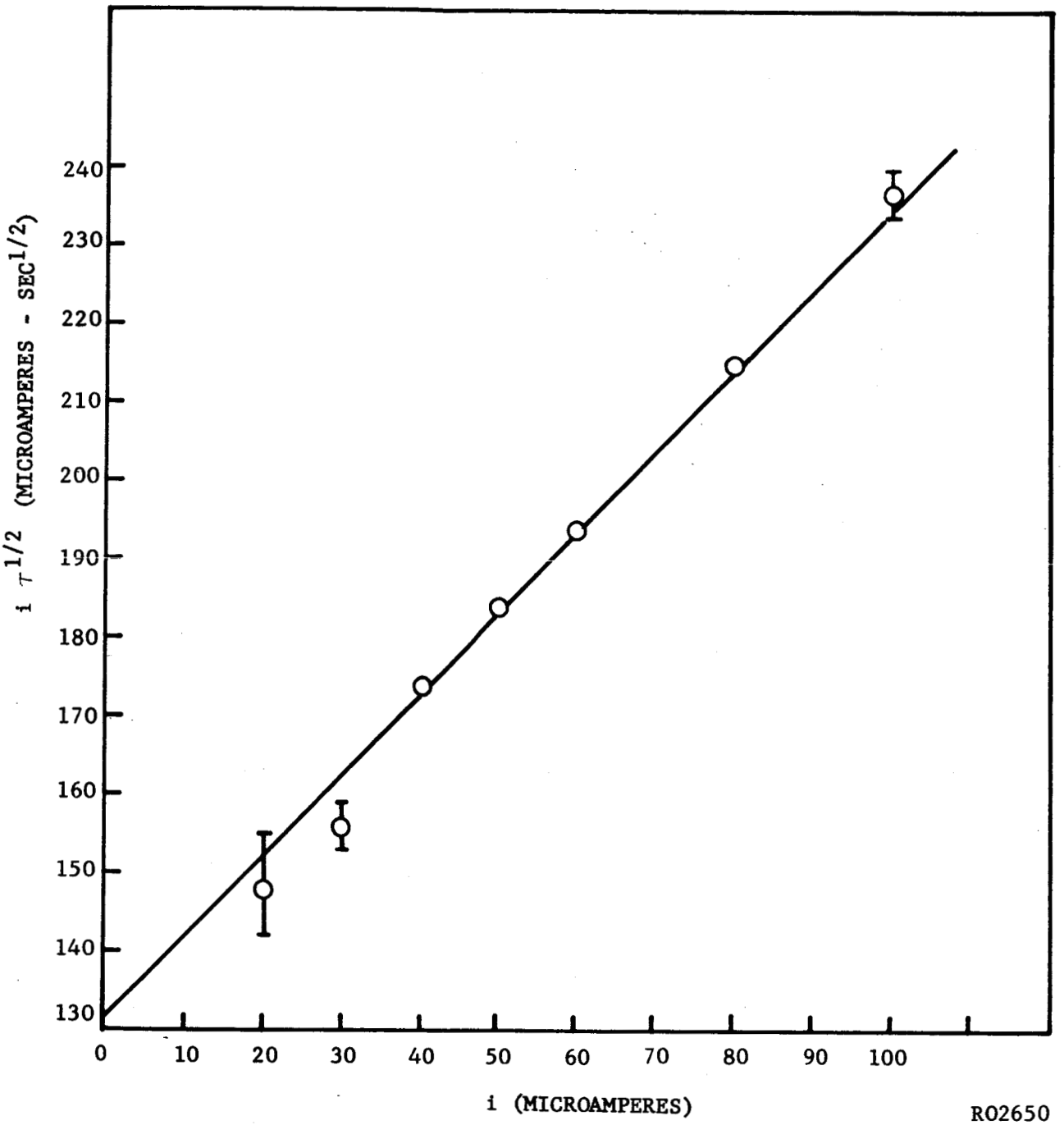

FIGURE 34. VARIATION OF $i\tau^{1/2}$ VERSUS i FOR 5×10^{-3} M TRYPTOPHAN IN UNSTIRRED 0.1 M $Na_2P_2O_7$ BUFFER SOLUTION

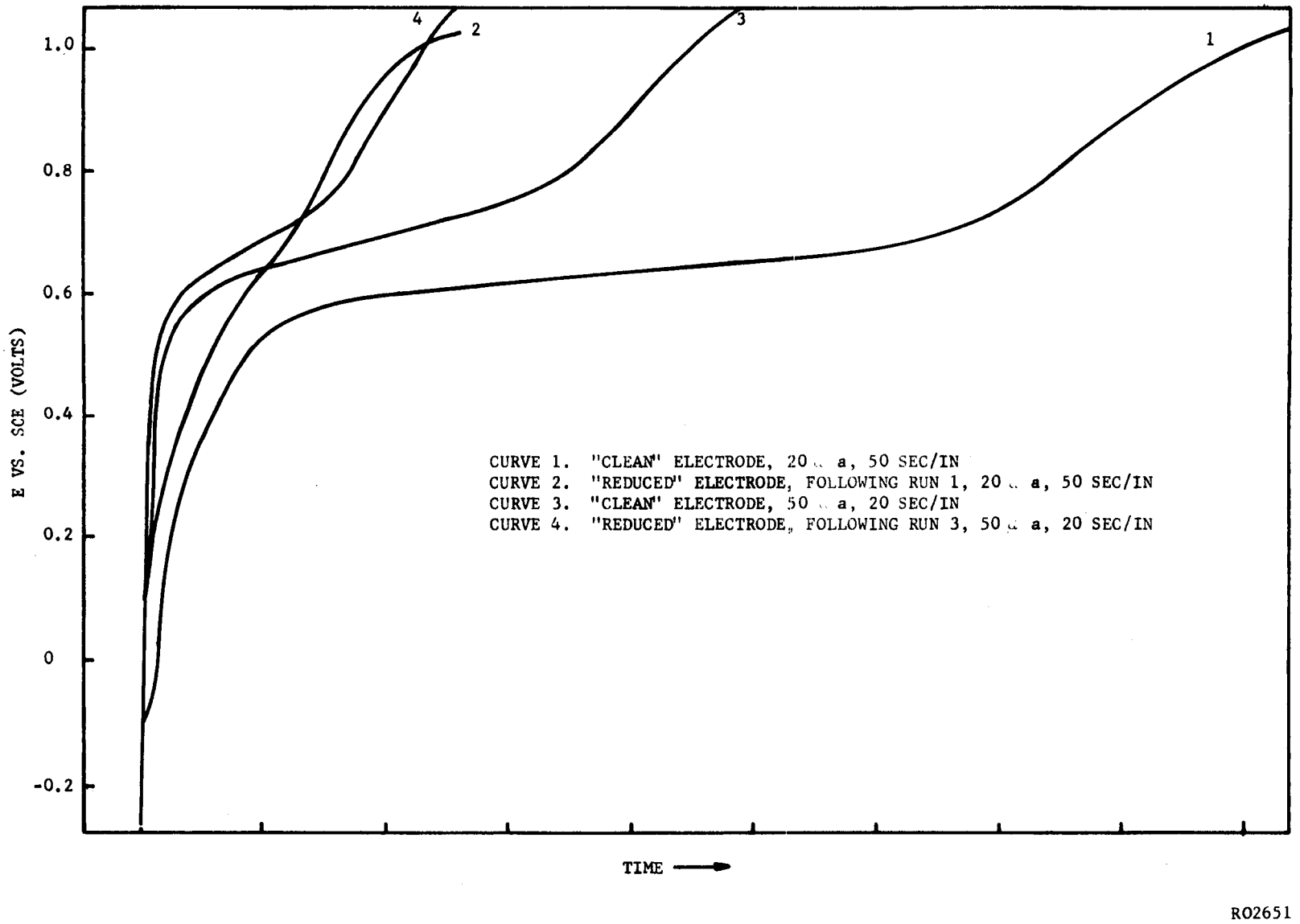

FIGURE 35. TYPICAL ANODIC CHRONOPOTENTIOGRAMS OF 5 x 10^{-3} M IPA - 0.1 M $Na_4P_2O_7$ SOLUTION ON "CLEAN" AND "REDUCED" ELECTRODE

TABLE 3

CHRONOPOTENTIOMETRY OF INDOLE-3-PYRUVIC ACID

$i, (\mu a)$	$\tau, (sec.)$	$i\tau^{1/2} (\mu a\text{-}sec.^{-1/2})$
25	167	324
30	134	348
35	89	328
40	74.4	346
50	51.6	360
60	28.8	322

Ave: 338 ($\mu a\text{-}sec.^{-1/2}$)
Std. deviation: 15.5

The other point of interest was the change in oxidation potential with time in freshly prepared solutions of IPA. This is apparently related to the change in structure of the IPA upon solution in a buffer, as outlined in the previous section. The effect is illustrated in Figure 36 where curve 1 shows a long potential halt, beginning at -0.13 v (SCE), for the freshly dissolved IPA, while subsequent curves show much reduced inflections at this point and, in curve 3, the inflection has practically disappeared. In these experiments, electrode poisoning was avoided by maintaining the electrode polarizing potential below 0.9 v (SCE) since it had been discovered that there was no inactivating effect below this voltage in the presence of either IPA or tryptophan. The rate of change in the length of the potential arrest at the low potential corresponds fairly closely to the rate of disappearance of the "enolic" form of the IPA in spectrophotometric measurements. Thus, it seems reasonable to assume that the changes in available current and the changes in the oxidation potential for IPA are due to the same factor, alteration in the relative amounts of keto and enol forms of the pyruvate derivative.

The complete DAO-tryptophan system was also investigated with chronopotentiometric techniques to determine the function of the enzyme in producing electroactive material. Figure 37 compares the chronopotentiometric oxidation of tryptophan alone (curves 1 and 2) for unstirred and stirred solutions, respectively) with tryptophan plus DAO (curves 3 and 4) to show that, under anaerobic conditions, the enzyme causes no change in the potential-time curves. In this and subsequent figures showing chronopotentiograms, differences in the point of origin of the curves are imposed electronically and do not represent a difference in electrochemical behavior. The data suggest that either the reduced enzyme cannot be oxidized electrochemically (adequate time was allowed for complete reduction of the enzyme) or that the enzyme was not present in high enough concentration to enable detection of any potential arrest due to such electrochemical oxidation. Under aerobic conditions, however, a stirred solution resulted in a continued oxidation at constant potential (curve 5) with the potential varying according to the polarizing current.

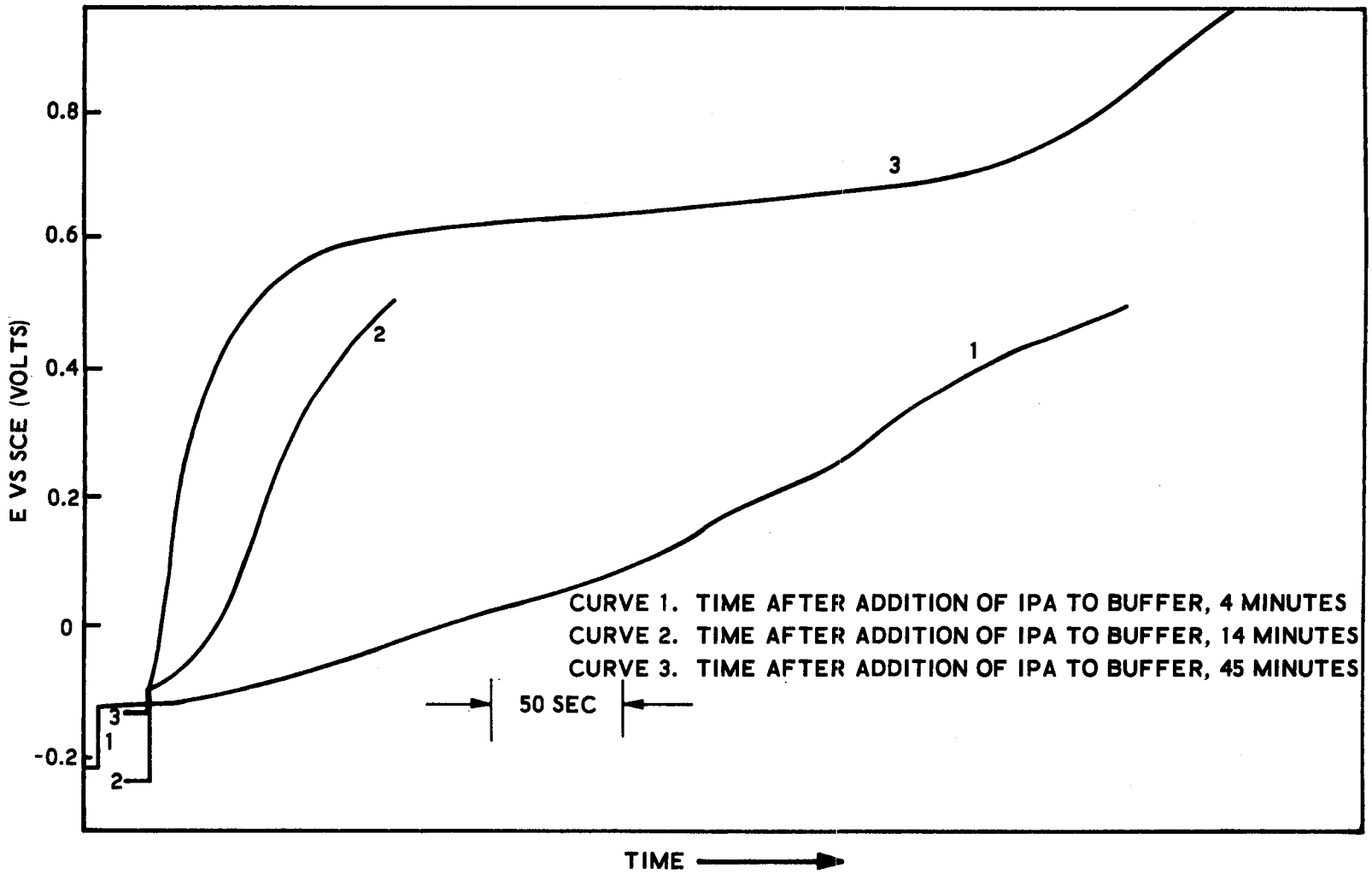

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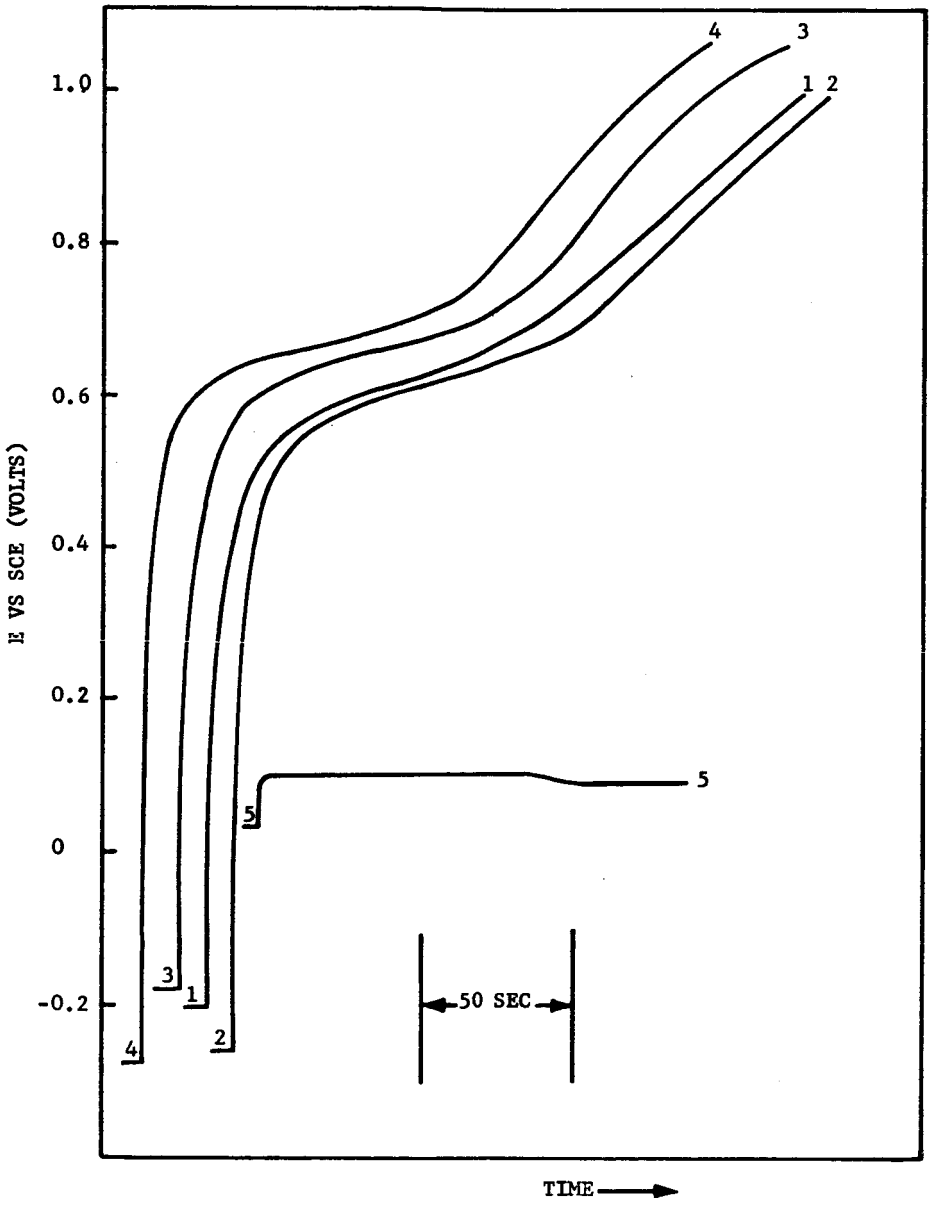
FIGURE 36. ANODIC CHRONOPOTENTIOGRAM OF 5×10^{-3} M INDOLE -3 PYRUVIC ACID IN 0.1 M $Na_4P_2O_7$ BUFFER SOLUTION OF pH 8.3, 20 a, SOLUTION UNSTIRRED



CURVE 1. 5 x 10^{-3}M D-TRYPTOPHAN ONLY, UNSTIRRED SOLUTION
CURVE 2. 5 x 10^{-3}M D-TRYPTOPHAN ONLY, STIRRED SOLUTION
CURVE 3. 1 ML D-AMINO OXIDASE ADDED TO 25 ML OF 5 x 10^{-3}M TRYPTOPHAN SOLUTION, UNSTIRRED SOLUTION
CURVE 4. 1 ML D-AMINO OXIDASE ADDED TO 25 ML OF 5 x 10^{-3}M TRYPTOPHAN SOLUTION, STIRRED SOLUTION
CURVE 5. 1 ML D-AMINO OXIDASE ADDED TO 25 ML OF 5 x 10^{-3}M TRYPTOPHAN SOLUTION, SOLUTION EXPOSED TO ATMOSPHERE, STIRRED SOLUTION

FIGURE 37. CHRONOPOTENTIOGRAM OF D-TRYPTOPHAN - D-AMINO ACID OXIDASE IN 0.1 M $Na_4P_2O_7$ BUFFER SOLUTION, 20 μa

A similar set of experiments, designed to quantify the effects of air on the formation of the reactive products, is shown in Figure 38. In addition to the anaerobic comparisons of tryptophan alone with tryptophan plus DAO in stirred and unstirred solutions (curves 1 through 4), tests were made upon the DAO-tryptophan mixture with a measured quantity of air of 0.05 ml (curve 5) and 0.10 ml (curve 6) bubbled into the system. It may be observed that 0.10 ml of air has a significant effect in lowering the potential of the plateau for the system. Thus, it is demonstrated once more that aerobic conditions are required for the formation of the electroactive material in the DAO-tryptophan system. It is of particular note, however, how little oxygen is required to produce significant amounts of electroactive material. Rough calculations would indicate that 0.1 ml of air would only introduce about 1 micromole of oxygen, allowing formation of 1 micromole of IPA from tryptophan oxidation, if it were entirely used. This, in the 25 ml of test solution, would amount to about 4×10^{-5} M IPA. The current supporting capabilities of such a small amount of IPA may have significant implications on the mechanism of action of the enzyme, DAO, since it is difficult to conceive that, if the keto structure were formed first, any significant currents would be found, in view of the very low amount of the enol in the equilibrium mixture, at pH 8.3. This would indicate that the compound released into the solution by the enzyme corresponds to the enolic structure. It therefore conflicts with some existing theories on the reaction sequence for amino acid oxidation by DAO, which require formation of the keto acid first.

4.6 STUDIES OF DAO IN HIGHLY CONCENTRATED SOLUTIONS IN AN EFFORT TO DETECT DIRECT REACTION OF THE ENZYME WITH THE ELECTRODE

As indicated above, DAO, in itself, did not affect the chronopotentiometry of tryptophan unless oxygen was introduced to allow formation of the oxidized products. Since DAO, in such experiments, was probably at concentrations of the order of 10^{-8} to 10^{-7} M at most, it would not be expected that the direct reaction of the enzyme could be observed with the techniques used. Accordingly, an effort was made to obtain conditions wherein the enzyme would be present, in the reduced state, at concentrations which would allow observation of any direct reactions. Highly purified enzyme was prepared. This enzyme was tested in the electrode compartment of the separated compartment cell in Figure 3. The solution used in the electrolyte space was 0.25 M D-tryptophan in pH 8.3 pyrophosphate buffer with sufficient bovine albumin (crystalline) added to maintain equal osmolarity due to nondiffusable protein on each side of the membrane. (Further details on use of this cell are given in Section V.) Sufficient time was allowed after introduction of the substrate solution and the enzyme into the cell to permit the substrate to diffuse into the enzyme compartment and obtain completely reduced enzyme (reduction could be observed by color change). Changes in potential were observed during this period and then the circuit was closed and current measurements made at 0.200 v (SCE). Comparisons of currents so obtained with those found prior to the reduction of the enzyme showed that there was no difference due to enzyme reduction even when the enzyme was present in concentrations up to 6×10^{-4} M. Thus, no evidence for any type of direct reaction could be obtained.

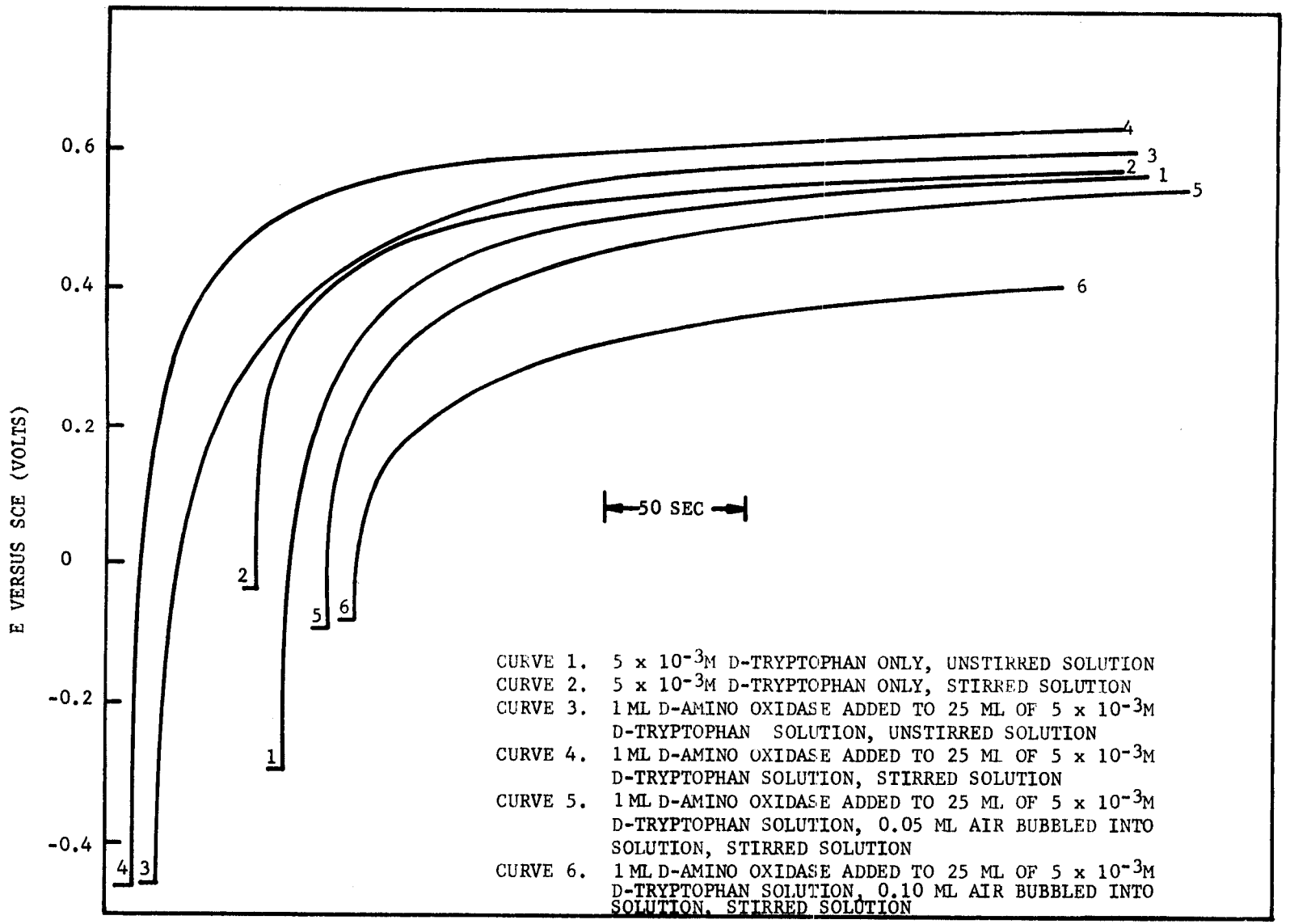


FIGURE 38. CHRONOPOTENTIOGRAM OF D-TRYPTOPHAN - D-AMINO ACID OXIDASE IN 0.1 $Na_4P_2O_7$ BUFFER SOLUTION, 5 a

4.7 CONCLUSIONS

The data given above support the conclusion that the electrochemical reaction in the DAO-tryptophan system is the result of aerobic oxidation of tryptophan to IPA with an electrochemical oxidation of the IPA to undetermined products. The reactive form of the IPA is probably the enol (supported by a variety of data) and the attack may be upon a quinoid form resulting from charge separation structures. This latter observation is based upon the failure of such materials as phenylpyruvate to undergo oxidation. Similar mechanisms probably obtain in related systems (tyrosine oxidation). All efforts to detect any direct reaction of the enzyme with the electrode have given negative results, although certain ambiguous results have been obtained with some experiments which would permit an interpretation that the enzyme affects the electrochemical reaction in some instances. Thus, enzyme added to an IPA plus tryptophan mixture, gives a slightly higher current value than the IPA-tryptophan alone, despite rigid efforts at excluding oxygen which might allow formation of enough additional IPA to give the observed results.

The results with DAO are notable in that they indicate that the oxidative enzymes may frequently give rise to products which are electroactive in contrast with the more reduced substrates. Thus, although some potential energy may be lost through this mechanism, aerobic cells could be developed based upon such selective reactions. Also of note is the need for complete definition of the system actually being studied, as was also brought out in the urease reaction.

Studies here also indicate that many biological systems may be unsuitable for consideration in biocells where a possibility of high polarizing potentials may occur. Tryptophan, IPA or DAO were all found to cause electrode poisoning when electrode potentials rose to 0.9 v or higher.

SECTION 5

EFFECTS OF ATTACHMENT OF ENZYMES AND ORGANISMS TO ELECTRODE SURFACES

The potential value of having organisms or enzymes attached to or incorporated in the electrodes may arise from one or more of these possible effects: (1) shorter diffusion paths between electrode and the biological reactive sites; (2) possible direct interaction between the organized structure of the cell (or enzyme layer) and the electrode, providing a direct electron transfer from the biological material to the electrode, either from within the cell or by direct transfer from an enzyme; (3) potential gradient effects and ion exchange centers due to the electrical double layers and sequestered compartments arising from the multimembrane systems of the organisms. The latter effects could give rise to direct diffusional flow of substrates, reaction intermediates and products as a consequence of potential gradients acting upon charged compounds.

Detrimental consequences might also be expected from attachment of organisms since there is the possibility of masking reactive electrode surface, interfering with diffusion and providing competing adsorption surfaces.

Experimental techniques which will allow direct determinations of single variables are difficult to visualize since any type of layering on the electrode surface is likely to introduce, to some degree, more than one effect. The approaches here were of two types. In one method, the enzymes or organisms were attached to the electrode surface by incorporating them into a gel matrix which was capable of permitting diffusion of the substrates and products but not of the enzyme or organism. Comparisons were then made of efficiencies and rates of reaction of the uncoated electrode with an electrode coated; in one case with the enzyme incorporated in the coating at the same amount or concentration as in the bulk solution; in a second case coated with a layer of the matrix material, without the biological agent. This approach has some obvious difficulties in that an enzyme may be oriented in the matrix in such a way as to be inactive (due to steric hindrance) and it is impossible to completely determine

the diffusional hindrance of the gel layer in the working situation although an approach may be made through the study of rates of diffusion of small, electroactive molecules to the electrode from the bulk solution. Obtaining sufficiently thin layers of coating for study of the reaction close to the electrode is also difficult.

The second approach was to use a compartmented cell which allowed the enzyme, or organism, to be retained in a thin layer in close proximity to the electrode by a semipermeable membrane. The anolyte bulk solution was contained in a second compartment and reaction occurred by diffusion of the enzyme substrate to the enzyme-electrode compartment where the anode reaction could also occur. Although the enzyme layer thus obtained would be relatively thick, the enzyme-electrode compartment was fully stirred so that diffusion across the enzyme layer was eliminated as a variable factor. The anode reaction would then be subject only to the limitations of diffusion across the electrode boundary layer and the concentration of the electroactive species in the electrode compartment. Numerous problems still arise. Diffusion rates through the separating membrane becomes a complicating factor and back diffusion of the enzyme reaction products (possibly the electroactive material) may also occur. The potential gradients are not as significant a factor as in the gel layer approach due to the mixing in the electrode compartment.

Combinations of these techniques could be used to obtain further evaluation of the magnitudes of the various effects in the coated electrodes. Use of neutral gels in comparison with anionic and cationic gels, or including various macromolecular polyanions or polycations in the electrode compartment could assist in determining the effects of ion exchange sites in specific reactions. Added compartments or a compartment with a gel covered electrode could also be used in evaluating the effect of multiple layers of membranes, such as would arise in the case of electrode adsorbed microorganisms. The work here has been largely limited to initial studies of the gel incorporated enzymes and organisms using agar as the matrix material, and to the evaluation of cell characteristics of the compartmented cell for attachment studies.

5.1 INCORPORATION OF ENZYMES IN GEL LAYERS ON THE ELECTRODE SURFACE

Although a number of possible supporting matrix media were tested for attaching enzyme to the electrode surface, only agar gels were found to be reasonably consistent for our studies. Acrylic resins were too highly resistant, polyvinylchloride preparations tested were unstable in the cell unless sufficient setting time was allowed (several hours); gelatin was impure and gave rise to spurious currents, in addition to being relatively unstable and slow in setting; other materials required the use of solvents which would inactivate the enzymes (true also of acrylic resins). Therefore, with the limited time available, all the gel studies were done with agar as the supporting medium.

Two types of electrodes were used in the studies. Most were done with the normal, flat platinum electrode, having a total area (both sides) of 4.0 cm. Also used was a coil of platinum wire which had the advantage of stabilizing the

gel layer better so that the enzyme-gel was not lost as frequently during the course of the experiment. The coil has a disadvantage in having an indeterminate surface area (studies with ferrocyanide and other reducing agents indicated that its effective reaction area was almost identical to that of the flat electrode) especially with an agar gel layer attached. The gel thickness would also be variable on this electrode. Certain characteristics during use were also different, as, for example, the current surge which would develop for a very short time after first closing the circuit.

Some of the first experiments with agar coated electrodes are given in Table 4. For these experiments a mixture of agar, buffer and enzyme was prepared (held at 47°C, enzyme added just before placing on the electrode) and the electrode dipped into viscous solution. The amount of film deposited was determined by weighing the electrode. After a short incubation in buffer solution to remove any diffusable enzyme, the electrode was tested in the electrochemical cell. It is apparent, from these measurements, that such a surface layer is capable of producing small currents but not at the same density as the free solution. The measurements in part B, with the enzyme in free solution but with an agar layer over the electrode show that the agar does permit diffusion of the IPA, the electroactive material, to the electrode. But part C, where the agar stripped electrode is tested in the same solution as in B, demonstrates that the agar had exerted some restrictive influence on the diffusion in B. It must be considered, in evaluating these experiments, that the enzyme concentration in the agar is seven times as great as it is in the bulk solution but also that the amount of enzyme used in the agar is only one-hundredth that used in the bulk solution.

Since diffusion through the agar appeared to be a restrictive factor, experiments on the rate of diffusion and effect of the agar on the simple oxidation of IPA were performed. As shown in Figure 39, diffusion through a thin layer (25 mg) of agar is a severely limiting factor. The initial oxidation rate is high but drops rapidly in each case to a much lower value indicating that IPA concentration in the vicinity of the electrode is decreasing rapidly on closed circuit operation due to the diffusion barrier and re-establishing the concentration at the electrode during the open circuit breaks.

Further coating studies were performed with a different type of enzyme coating procedure. Solid enzyme, commercial DAO, was weighed and distributed over the surface of the electrode; then the agar-buffer, at 47°, was poured over the solid material to hold it in place. This approach was used to enable working with the cruder enzyme. Comparisons were again made between electrochemical reaction with this enzyme agar mixture and the reaction obtained with enzyme in the bulk solution. Tests were made using a carrier, in these cases, rather than being limited to the formation of the electroactive IPA. Thus, ferricyanide was incorporated into the bulk medium to permit an efficient anaerobic reaction to develop and eliminate the complicating factor of oxygen diffusion into the agar. Once in the agar, the ferricyanide should act as a cyclic-oxidizing agent (not possible with oxygen) so that only a short diffusion path would be required in the attached enzyme system.

TABLE 4

ENZYME INCORPORATION IN AGAR COATINGS ON ELECTRODES

Conditions:

A. Enzyme in agar. 0.5 ml DAO, 0.02 ml catalase, 0.5 ml P-P buffer in 1 ml 2% agar, 17 mg. of mixture (equivalent of 0.005 ml DAO) coated on coil electrode (3.54 cm total surface) tryptophan, 0.005M, in P-P, pH 8.3 buffer.

B:1 Enzyme in free solution, agar coated electrode. Electrode coated with 17 mg agar mixture as above with buffer replacing enzyme. 0.5 ml DAO, 0.1 ml catalase in tryptophan-buffer solution used above.

	Condition	Atmosphere	V_o(mv)*	V_c(mv)*	I_c(ua/cm^2)*	IPA** Mx10^3
A.	Enzyme in Agar	N_2	90	Unstable	Unstable	0.05
		Air	85	240-290	2.5	0.1
B.	Enzyme in Solution Electrode Agar Coated	N_2	110	200	2.7	0.1
		Air	-	200	6 → 20	1.4
C.	Enzyme in Solution	N_2	-	200	55	1.5

* V_o - open circuit potential

V_c - closed circuit potential

I_c - load current

** Apparent indole-3-pyruvic acid concentration determined spectrophotometrically

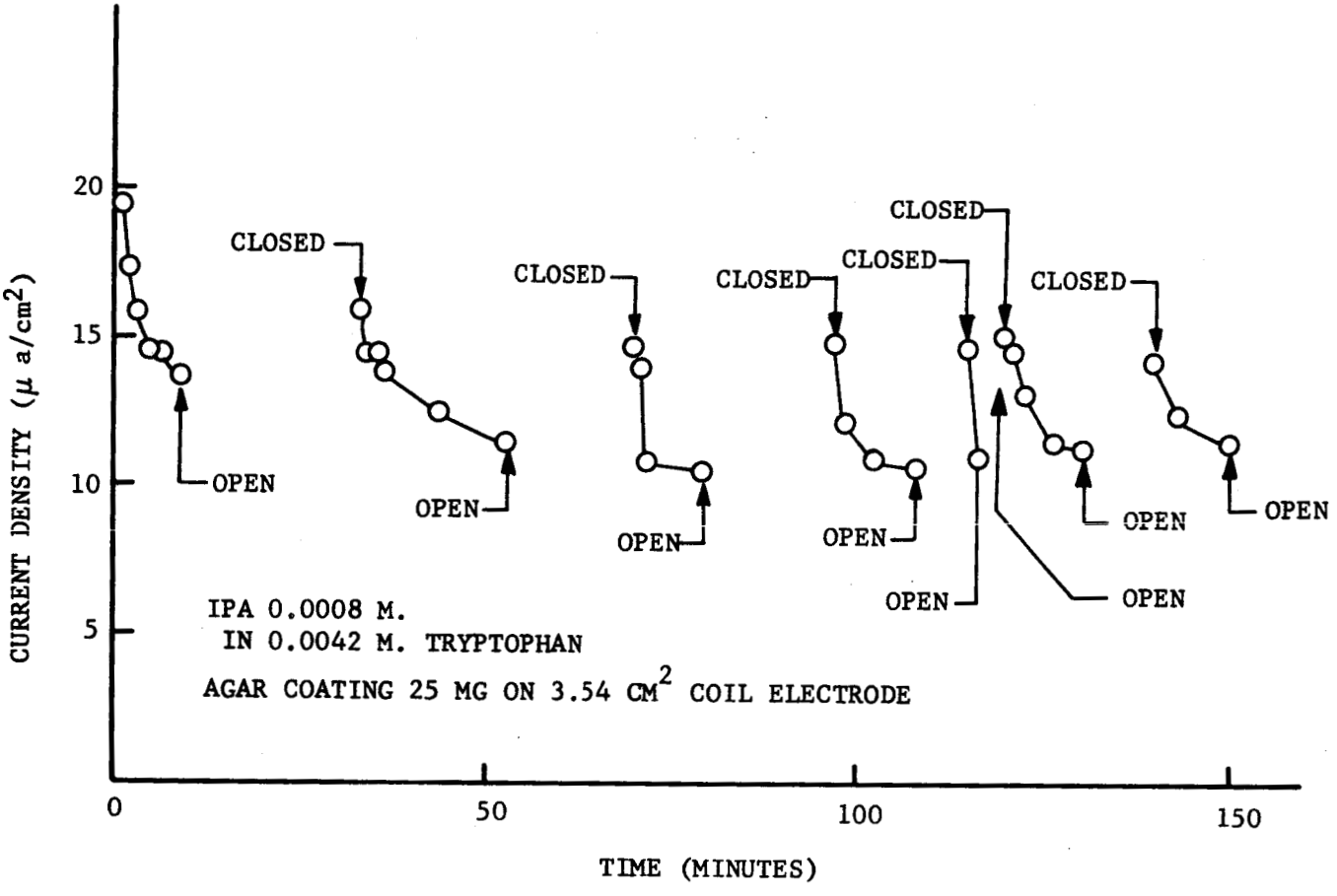


FIGURE 39. EFFECT OF INTERMITTENT RECOVERY PERIODS ON CURRENT FROM IPA WITH AGAR COATED ELECTRODE

Results of some of these experiments are given in Table 5. The values given for current development are those obtained after 10 minutes of development which was adequate to obtain a reasonably constant current (normally still rising slightly) for most experiments. The current per unit weight of enzyme is based upon the weight of enzyme present in the space corresponding to the agar layer. This layer was generally about 200 to 250 mg for the entire electrode and so the effective enzyme in the bulk solution has been assumed to be that enzyme present in the 0.25 ml of solution adjacent to the electrode (1/100 of the total enzyme present in the entire solution). It may be seen that the total current developed within the experimental period is generally fairly high for the total amount of enzyme used in the agar layer in comparison with the currents found for the enzyme in the bulk solution. However, on the basis of the enzyme in the immediate vicinity of the electrode, which is the actual effective enzyme for the purposes here, the agar incorporated enzyme suffers in comparison. The incorporation of the enzyme does seem to have one highly significant advantage. It is apparent, from the experiments in which the enzyme reaction occurred in the bulk solution with an agar layer on the electrode, that the agar formed a barrier to the reduced products of the enzyme-carrier reaction. Yet, when the reaction occurs in the agar, itself, there is a reasonably effective transfer. It is quite possible that the efficiency of the agar incorporated enzyme might be much higher were the enzyme in a much thinner layer with commensurately lower diffusion resistance. Thus, it appears that, under present conditions of test, the free solution enzyme has a greater efficiency than the coated enzyme but a question still remains as to whether this order would exist under more optimal conditions of coating.

5.2 ATTACHMENT OF MICRO-ORGANISMS ONTO THE ELECTRODE

The techniques used in study of the effect of attaching micro-organisms on the electrode were similar to the procedures used in the experiments cited in the last paragraphs. E. coli was grown in large batch quantities and harvested by centrifuging. A heavy suspension of the cells were applied to one side of the electrode and weighed. Agar was then added on top of the cells to act as a support. Tests were then made on a similar basis, comparing the agar incorporated cells with a similar quantity of cells in the bulk medium with and without agar on the electrode. One form of data presentation on these experiments may be found in Table 6. Once more, it may be seen that the current values at a given time with the attached bio-electrode are higher than those for the corresponding time with a similar amount of cells in the bulk medium. However, when the calculations are made on the basis of the cells in the immediate vicinity of the electrode (assumed volume of 0.25 ml) the free bacteria are much more efficient than the immobilized cells. It is apparent that the thickness of the agar coating affects current adversely. In Figure 40, the same experiments are presented to show how the agar incorporated bacteria cause a more rapid initial development of current but the free cells soon overtake the immobilized cells in forming the electroactive material. Again, as with the enzymes, agar coating the electrode caused extreme inhibition of the bulk solution electrode reaction.

Thus, attached organisms, under the conditions observed here are of lesser efficiency than the free organism. However, as in the case of enzymes, the possibility of a much more efficient reaction in better defined layers cannot yet be excluded.

TABLE 5

COMPARATIVE EFFICIENCY OF BIOELECTRODES USING D-AMINO ACID OXIDASE IN BULK SOLUTION OR ATTACHED TO ELECTRODE

All experiments used 25 ml of pH 8.3, 0.1 M pyrophosphate buffer containing 200 mg. D-alanine and 0.001 M potassium ferricyanide with anaerobic measurements were all made at 0.30 v polarizing potential conditions.

A - Attached enzyme. The indicated quantity of commercial DAO was weighed and fixed to the electrode face with agar.

B - Enzyme in bulk medium. The indicated quantity of enzyme was added to the buffer-alanine solution. No coating was used on the electrode.

C - Enzyme in bulk and electrode coated. Enzyme was added as above and the electrode was coated with agar to the same degree as used for the enzyme support in A.

	Total Enzyme (mg)	Total Current (μa)	Current per* mg enzyme at electrode
A - Attached Enzyme	6.7	5.25	0.78
	10	8.5	0.85
	15	9.2	0.61
	25	9.6	0.38
B - Bulk Enzyme open electrode	25	0	-
	50	4.8	9.6
	100	13.9	13.9
	200	19	9.5
C - Bulk Enzyme coated electrode	50	0	0

* Current based upon the amount of enzyme in the volume equivalent to the volume of the agar layer.

TABLE 6

COMPARATIVE EFFICIENCIES OF E. COLI IN BULK MEDIUM AND IMMOBILIZED AT THE ELECTRODES

E. coli paste freshly harvested. All determination made using C medium (6) as fuel and electrolyte, 25 ml. Ferricyanide, 10^{-3} M, added as a mediator. Polarizing potential at 0.30 v during current measurements. Anaerobic condition throughout.

A - Immobilized organism. Indicated quantity of cell paste spread on electrode and then held in place by addition of indicated amount of agar, applied at 45 to 50°C.

B - Bulk medium organism. Indicated amount of E. coli added to electrolyte solution. Electrodes not coated.

	Agar (mg)	E. Coli (mg)	Current* (μa)	Current per ** mg E. Coli
A - Immobilized organisms	68	22	510	23
	98	18	490	30
	100	27	410	15
	109	26	435	16.7
	128	26.5	385	14.5
	157	35	300	8.6
	293	29	210	7.2
B - Organism Solution		19	250	1300
		24	250	1040
		24	335	1400
		26	250	960
		26.5	290	1100
		28	260	930

* Current obtained after 10 minutes of operation of cell after closing circuit.

** Current per unit of "effective" organism based on E. coli in volume equivalent to the volume of the agar layer.

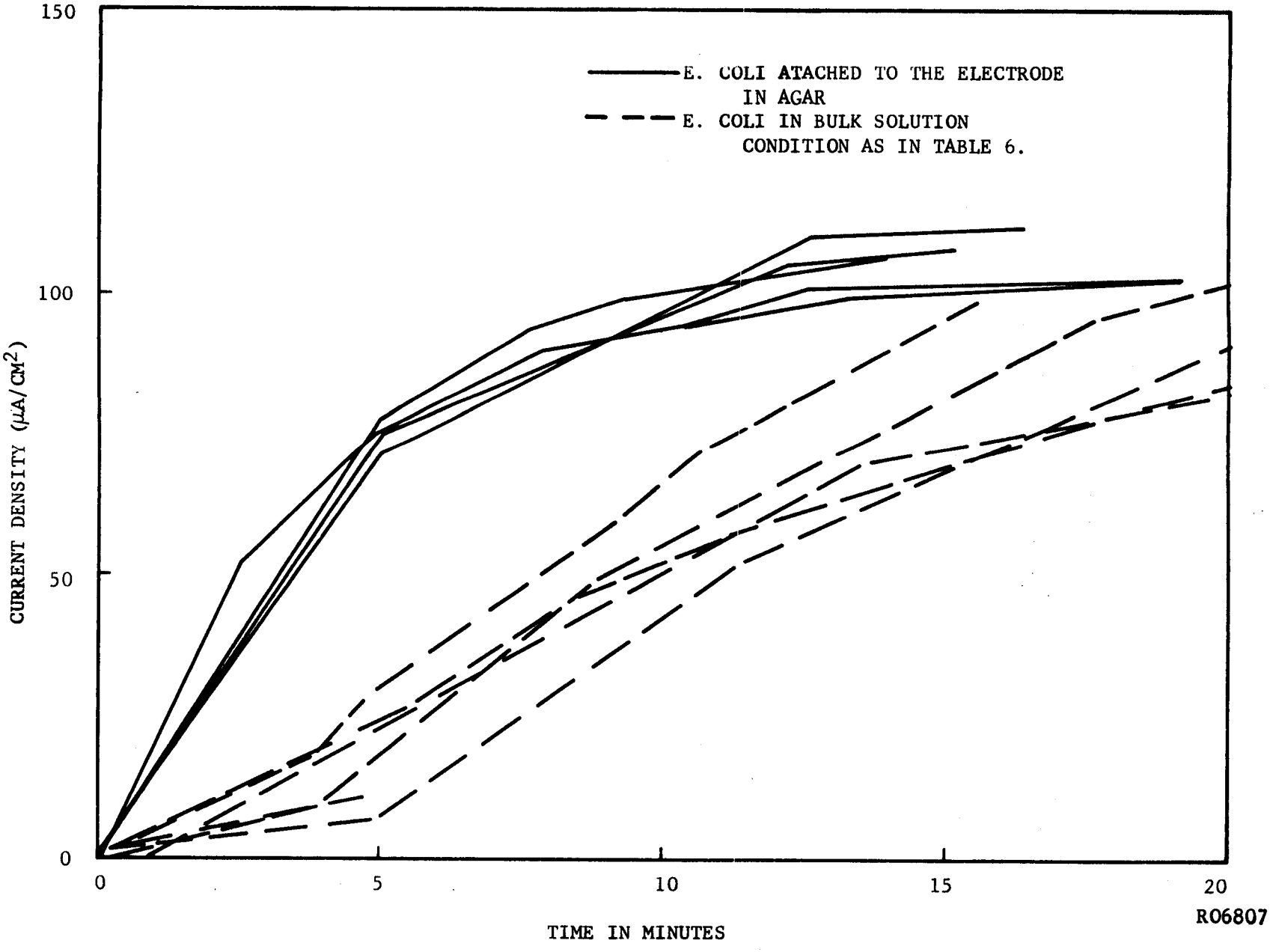


FIGURE 40. E COLI BIOANODE

Incorporation of platinum filings into the agar, in an attempt to obtain an electrode extending into and around the bio-system, did not improve action either with the organisms or enzymes.

5.3 TESTS OF THE MULTI-COMPARTMENT CELL FOR STUDIES IN ENZYME AND ORGANISM ATTACHMENT

The apparatus in Figure 3 was designed to enable the study of enzymes and organisms in free solution or suspension under circumstances analogous to those obtaining with the biological agent attached to the electrode. In principle, it simply retains the enzyme or organism at the electrode surface (through the agency of a semipermeable membrane) while allowing access of the substrate (or electro-active material) from the main anolyte chamber by diffusion through the membrane. In the latter effect, it is analogous to the agar system which requires diffusion across an unstirred layer. However, in the cell there will be little or no potential or diffusion gradient across the reaction space since it is under constant vigorous stirring.

It was essential, before attempting experiments with the enzyme systems, to obtain some knowledge about diffusion characteristics across the membrane into the reaction area and the effects of continued electrolysis during such diffusion. To test the diffusion, the rates of development of current were tested with ferrocyanide and hydroxylamine after adding these compounds in known concentration to the anolyte chamber.

Results of some tests with hydroxylamine are given in Figure 41. Two types of measurement were used, the first with a continuous current at 0.20 v (Curve 1) so that hydroxylamine was being used constantly as it entered the cell. This had the effect of maintaining a higher concentration difference on the two sides of the membrane over a longer period of time. With this type of measurement it should be possible to obtain a direct measurement of diffusion rate by balancing the electrode potential to the point where hydroxylamine was oxidized at the same rate as it entered the electrode compartment, thus maintaining a constant current value (not done in these experiments). Curve 3 gives the results of the second procedure, essentially a free diffusion to equilibrium. The circuit was maintained open except for very short periods (2 to 3 seconds) each minute for measuring the change in concentration by determining the current at 0.20 v (SCE). Curves 2 and 4 show the electrode compartment concentration of hydroxylamine based upon previously determined calibration curves for current versus hydroxylamine concentration.

Ferrocyanide results were similar. The curves so obtained provide a definition of some of the diffusion characteristics of the compartmented cell. However, for each system studied, diffusion characteristics should be determined upon the substrates and products involved in the bioelectrode.

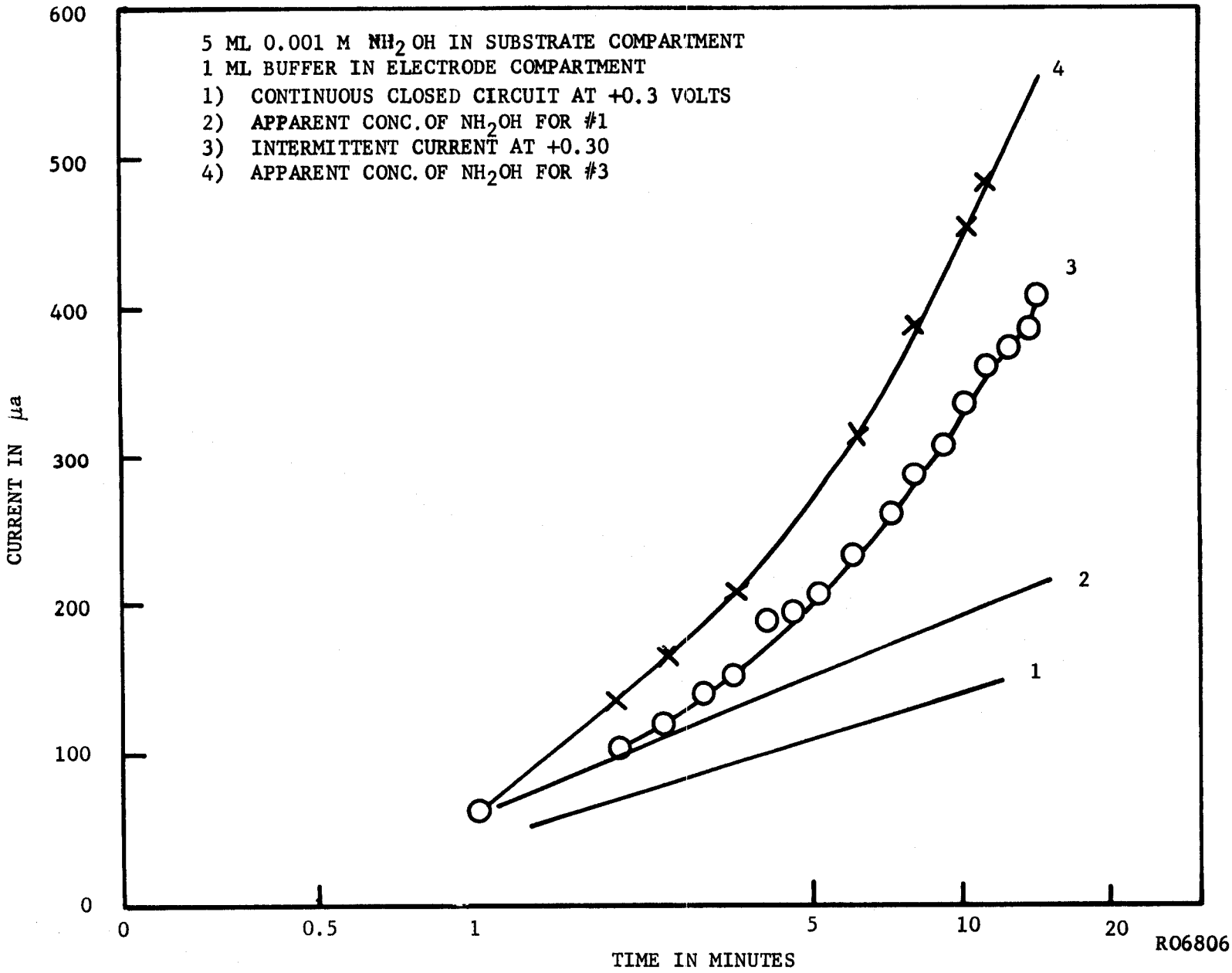


FIGURE 41. HYDROXYLAMINE DIFFUSION AND OXIDATION IN A MULTI-COMPARTMENT CELL

5.4 ENZYME TESTS IN THE COMPARTMENTED CELL

Short discussions of tests of the DAO-alanine system in the compartmented cell have been given in Section 4.6. The primary objective of these tests was the detection of any possible direct reaction of the reduced enzyme with the electrode so that they were not conducted in such a manner as to provide information upon the effect of compartmentation on diffusional and kinetic aspects of the DAO bio-electrode. Attention to tests with micro-organisms in connection with satisfying specific contractual points has limited time available to pursue this interesting area.

5.5 CONCLUSIONS ON ENZYME AND ORGANISM ATTACHMENT

The present means of attaching enzymes and organisms to electrodes are far from satisfactory. Much work remains to be done in obtaining mechanisms for adequate research treatment to determine parameters which affect performance. With the relatively crude procedures used here, some of the attached enzyme or bacterial systems appear to have a greater efficiency than the corresponding free solution systems, on the basis of developing currents and potential more rapidly than the free solutions. However, this is somewhat illusory since a treatment based upon the comparisons of amount of enzyme in the vicinity of the electrode, as would be necessary in a practical cell, demonstrates the free solution (or suspension) systems to be much more efficient by the order of 2 magnitudes.

As indicated, though, the present conditions are certainly not optimal for the attached bioelectrodes. It is quite possible that a bioelectrode with a suitably thin layer of active material, so as to avoid excessive diffusion barriers, and with proper organization, to eliminate steric problems, might still be highly efficient in comparison with the free solution system.

SECTION 6

USE OF MODEL MOLECULAR SYSTEMS IN THE STUDY OF THE REACTION OF BIOLOGICAL MACROMOLECULES AT ELECTRODES

The purpose of the micro multicompartment cell described previously is to provide optimum conditions under which to examine the possibility of existence of a direct reaction between the electrode and reduced enzyme. As indicated, there are still many problems associated with the use of the cell, or other systems, either for detecting the direct reaction or for determining the quantitative effect of other relations, such as the potential gradients and interactions between various biological macromolecules and the electrode system. One of the major problems to be faced is the lack of apparent reactivity of the normal biological catalysts and carrier systems with the electrode normally used in the electrochemical measurements. Other problems are associated with the difficulty in obtaining highly purified, well defined biological systems for adequate quantitative treatment.

Such difficulties are exemplified by the extremely disconcerting behavior of the urease system in the electrochemical cell.

It seemed of some importance to attempt to obtain systems wherein the various parameters could be well defined so that it would be possible to determine whether biological reactions were being limited by the reactivity of specific groups, steric hindrance or by the diffusional characteristics of the macromolecules. Thus, some efforts have been directed toward the synthesis of model redox molecules, having prosthetic groups with well defined oxidation potentials, of such a nature that they could be used to determine the effect of size and shape of a matrix, corresponding to the protein of the enzyme, upon the redox properties and the ability of the prosthetic group to react with an electrode. The major effort has been devoted to attempts at attaching a dye, of known redox properties, to a series of graded amino acid, peptide or protein matrices. Tests have also been conducted upon attachment of other molecules capable of being oxidized or reduced.

6.1 ATTACHING REDOX DYES TO AMINO ACIDS AND PEPTIDES BY DIAZO COUPLING

Diazo coupling for attaching complex aromatic groups to proteins is a method of long standing. Landsteiner (18) and others have made literally thousands of protein derivatives by this procedure for immunological investigations. Accordingly, it seemed to be the most adaptable to the requirements for the formation of a model redox enzyme system through attaching a redox dye of known characteristics. The general reactions for coupling of a dye with a protein are:

$$(1)\quad \text{Ø-NH}_2 + \text{HNO}_2 \xrightarrow{\text{HCl}} \text{Ø-N=N}^+\text{Cl}^-$$

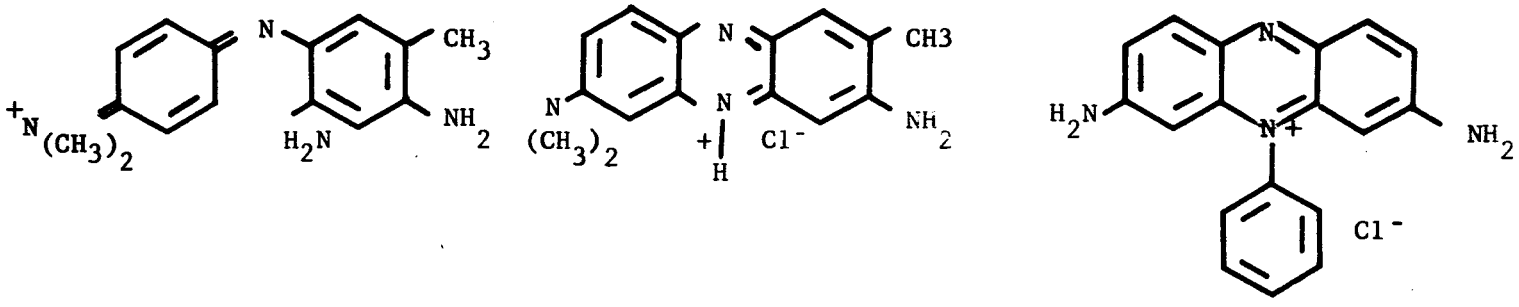
$$(2)\quad \text{Ø-N=N}^+ + \text{protein} \xrightarrow{\text{OH}^-} \text{protein - N=N-Ø}$$

Ø is an aromatic group which may be simple or complex and the attachment to the protein may occur either on the phenol group of tyrosine, in the position ortho to the hydroxyl, or on the imidazole group of histidine. The latter reaction is more readily accomplished and may occur selectively under some circumstances. Therefore, the major requirement appeared to be to obtain a redox dye, of a desirable oxidation potential range, containing an amino group which would permit the diazotization reaction and subsequent coupling reaction to occur.

Toluylene blue, an azo dye having an oxidation potential of the proper value and with the two amino groups (Figure 42, compound I) was tried first. Unfortunately, the two amino groups allowed intra-molecular reactions to proceed more rapidly than the intermolecular reactions so that it was impossible to obtain any coupling with this compound.

Neutral red (or toluylene red, Figure 41, II) was also tested, as was phenosafranine (Figure 41, III). Reaction conditions were essentially standard conditions for diazotization (19) involving the diazotization of the dye in solution with 3 to 4 equivalents of acid and the addition of one equivalent (or slightly more) of sodium nitrite while cooling in ice. This reaction mixture was then added to the coupling material, tyrosine, histidine or other phenolic material, with the coupling agent in excess in basic solution (pH 8 to 9, buffered with carbonate or strong buffers). Reaction products were separated by adsorption and elution from a DEAE cellulose column, eluting first with pH 7.6 phosphate buffer and finally with dilute sulfuric acid.

Reaction products were obtained from coupling between the phenosafranine and tyrosine, or histidine, as well as a number of other compounds, and for neutral red with several phenol derivatives (but not tyrosine). The phenosafranine-tyrosine product was tested for reduction with mild reducing agents and for reaction with enzyme systems as a carrier. The product was readily reduced by agents such as dithionite (hydrosulfite) but, upon re-oxidation by air, it was obvious from the color of the reoxidized solution, that the reduction had split the diazo bond involved in the coupling and regenerated the phenosafranine. Neutral red coupling products behaved similarly. Reduction of the coupled molecules preferentially reduced the diazo group, causing splitting. The use of phenosafranine and neutral



I

TOLUYLENE BLUE

E_o = 0.601

E_o'(pH7) = 0.115

II

NEUTRAL RED

E_o'(pH7) = -0.325

III

PHENOSAFRANINE

E_o'(pH7)= -0.220

$\phi - N = N - \phi$

DIAZO GROUP

E_o = 0.300

R06809

FIGURE 42. REDOX DYES USED IN DIAZO COUPLING EXPERIMENTS

red had been avoided, initially, due to the low oxidation potentials exhibited by these compounds in comparison with the oxidation potential of the diazo bond (see Figure 42 for oxidation potentials) which made it appear likely that diazo group reduction would occur preferentially. The toluylene blue, with a positive oxidation potential, might have been reduced without affecting the diazo coupling bond, if such a coupling had been possible.

Since the compounds tested comprise the readily available dyes with amino groups which might be diazotized, it becomes obvious that the initial attack will not serve to provide the desired reversibly oxidizable enzyme models. However, the use of the diazo bond, itself, should be tested as a reversible redox group. The presence of stabilizing, resonating groups adjacent to the diazo group can stabilize the reduced form of the group so that splitting does not occur under mild reducing conditions. Thus, with the choice of the proper aromatic grouping, it may be possible to attach a group to the protein or amino acid and then carry out reversible oxidations and reductions of the diazo bond used in coupling.

6.2 INVESTIGATION OF HYDROXAMIC ACIDS AS POSSIBLE REDOX GROUPS

Hydroxylamine and some of its derivatives, such as phenylhydroxylamine, are readily oxidized electrochemically. Therefore, it seemed worthwhile to investigate the possibility of making hydroxamic acid derivatives of amino acids, peptides, and proteins to serve as model redox systems. To provide a simple model for electrochemical tests, phthalic monohydroxamic acid was synthesized.

Synthesis was accomplished by mixing equimolar amounts of ethanolic solutions of phthalic anhydride and hydroxylamine hydrochloride and adding concentrated ammonium hydroxide to give a slight surplus. The hydroxamic acid began precipitating and was collected and recrystallized from ethanol-water mixtures. The ferric chloride reaction was used to confirm the hydroxamic acid structure. Tests of the recrystallized material electrochemically showed very little electrochemical activity at 0.3 v polarizing potential (Table 7). Therefore it was concluded that hydroxamic acids would not be suitable derivatives to use in the electrochemical investigation of redox protein models.

6.3 OTHER PROCEDURES

It is possible to attach hydroquinone or quinone carboxylic acid derivatives to proteins by formation of the amide with the lysine groups of protein (20). Time did not permit the testing of this method.

TABLE 7

ELECTROCHEMICAL OXIDATION OF PHTHALIC HYDROXAMATE

A. Pthalic hydroxamate, 2 x recrystallized, 0.003 M, in pH 8.0 pyrophosphate buffer

B. Phthalic hydroxamate, once recrystallized, 0.003 M, in pH 8 buffer

	Time (min.)	i(μa at 0.3 v)
A.	0	38
	2	33
	7	26
	13	22
	23	18
B.	0	--
	1	56
	2	49
	8	32
	13	28

SECTION 7

THEORETICAL CONSIDERATIONS OF THE POSSIBLE DIRECT REACTION OF AN ENZYME WITH AN ELECTRODE

One of the major points of interest underlying the present investigations has been the question of whether a significant improvement in the bioelectrode activity might occur as a result of a direct reaction of the catalyzing enzyme with the electrode itself, thus eliminating a further chemical reaction step following the enzyme-substrate reaction. The apparently improved activity found in bioelectrodes where the organism was in intimate contact with the electrode was presumed to derive partly from such a reaction mechanism.

However, a crude calculation of the probable value of an attached enzyme system, assuming a perfectly adsorbed monolayer of enzyme and with perfect transfer of electrons at a rate similar to the normal turnover rate of the enzyme in free solution, suggested that the current or power from such a mechanism might be vanishingly small (21): maximal current values of the order of microamperes per cm^2 of electrode surface were indicated.

A more comprehensive examination of this problem was therefore deemed to be desirable. However so many reactions; simultaneous, consecutive, and competing are involved in the operation of the bio-fuel cell that an exact analysis of the mechanism is out of the question. Fortunately, the capability of the cell as a power source can be evaluated quite adequately without knowing the detailed mechanism. The results will not be in terms of power actually produced, but of power which could be produced if: (a) a specified process (e.g., diffusion) is slow enough to limit the overall rate, and (b) all other processes are, if not instantaneous, at least faster than the process in question. If we consider every process which is likely to be slow enough to be the rate-determining step, and deduce the rate at which it must proceed, we can combine the results to deduce the intrinsic efficiency of the cell, i.e., the rate of the reaction-as-a-whole. Obviously, this is exactly the rate of the slowest of the processes considered.

This analysis is concerned only with the bio-fuel cell as power source. Its other function, as an essential component in a closed ecological cycle, is not here considered.

7.1 PRELIMINARY EXPLANATION

Since it has not been possible to obtain sufficient data on a single enzyme to enable analysis of the bioelectric problem, a composite enzyme has been selected with properties which could be considered representative for a number of oxidative enzymes. The model has the following physico-chemical properties:

(1) Molecular weight of 100,000.

(2) Spherical configuration of 60 to 65 $\mathring{A}$ diameter (the spherical configuration will give optimized results).

(3) A flavin (FAD) prosthetic group.

(4) An amino acid composition similar to that of myoglobin (see Table 8) but with a random sequence rather than following the myoglobin structure.

(5) Diffusion constant, D, with a value of 6.8×10^{-7} $cm^2 sec^{-1}$.

(6) A normal maximal reaction velocity similar to that of DAO, about 1000 reactions per minute per molecule of enzyme.

(7) A maximum permissible solution concentration of less than 5×10^{-3} M, equivalent to the approximate calculated concentration for the crystalline enzyme in a face centered cubic lattice.

(8) A reaction similar to that of DAO, oxidation of an amino acid or another compound which would have a similar free energy change upon oxidation.

TABLE 8

COMPOSITION OF PROTEIN AND DEDUCTION THEREFORM

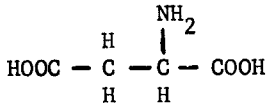
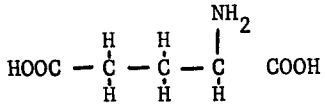
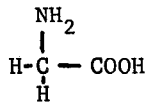
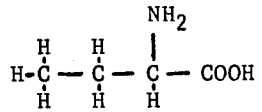
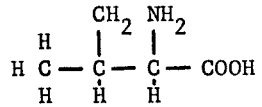
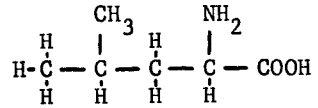
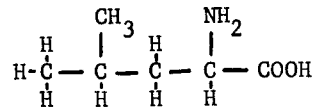
Group	Formula	Relative Number	Ionic Group	Molecular Weight	Number at Surface
Aspartic Acid		8	1 COOH	133.11	14.75
Glutamic Acid		19	1 COOH	147.14	35.02
Glycine		11	0	75.07	20.27
Alanine		17	0	105.14	31.33
Valine		8	0	117.15	14.75
Leucine		18	0	131.18	33.18
i-leucine		9	0	131.18	16.59

TABLE 8 (Continued)

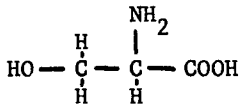
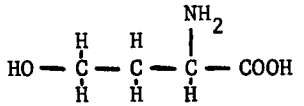
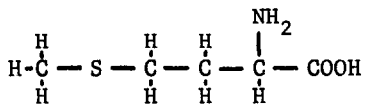
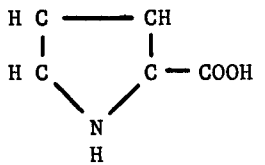
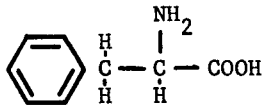
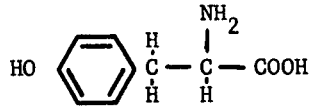
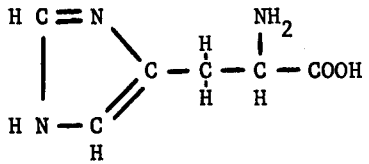
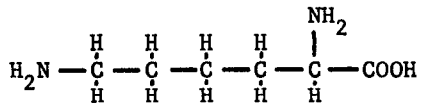
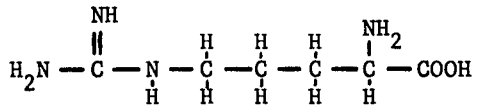
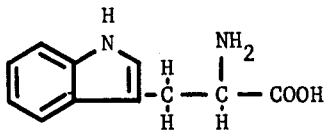
Group	Formula	Relative Number	Ionic Group	Molecular Weight	Number at Surface
Serine		6	1 OH	85.10	11.06
Threonine		5	1 OH	103.12	9.22
Methionine		2	0	149.22	3.69
Proline		4	0	111.10	7.37
Phenylalanine		6	0	165.20	11.06
Tyrosine		3	1ØOH	181.20	5.53

TABLE 8 (Continued)

Group	Formula	Relative Number	Ionic Group	Molecular Weight	Number of Surface
Histidine		12	2 N	155.16	22.12
Lysine		19	1 NH_2	146.19	35.02
Arginine		4	1 - NH_2 1 C=NH 1 NH	174.21	7.37
Tryptophan		2	1 NH	204.22	3.69

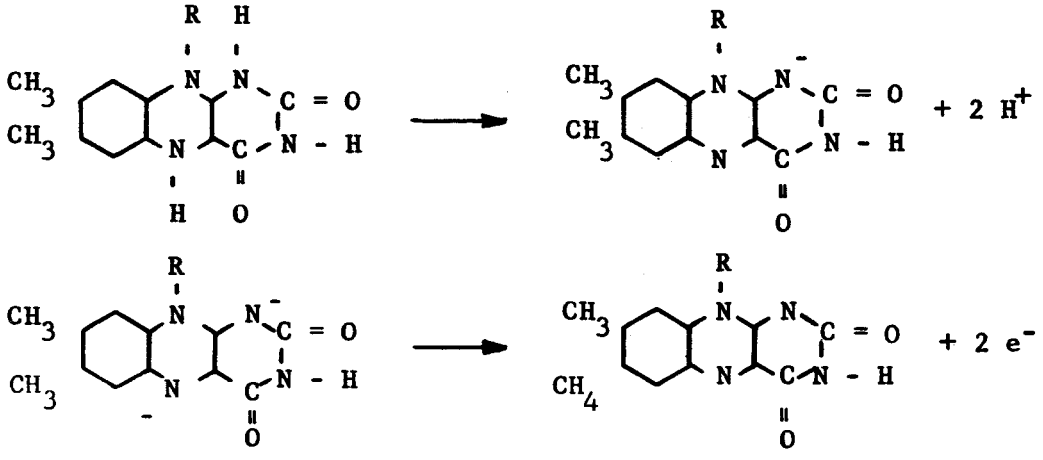
ΣnM = 19944.665 average M of amino acid residues = 112.341

Σn = 153

Molecular Weight Protein = Σ nM - 153 H_2O to form amide linkages

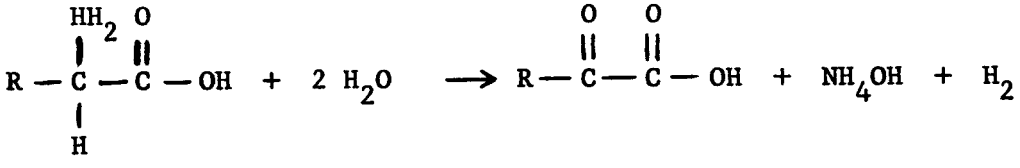
For M of protein 890 amino acid residues

The reaction is as follows:



$$2H^+ + 2e^- \longrightarrow H_2 \text{ (gas)} \qquad \text{(at cathode)}$$

When all substances which appear on both sides are cancelled out, the net reaction is:



The free energy change has a probable value of approximately 10 kilocalories per mole, from which the cell voltage on open circuit is

$$\epsilon = - \frac{\Delta F}{2\mathcal{F}} = 0.2169 \text{ volts}$$

The 2 in the denominator appears because two electrons take part in the oxidation.

This voltage is on the low side, but if the reaction is fast even when current is being drawn, and if the cell resistance is low, it does not preclude successful use as a power source.

It will be shown later that the adsorption of the enzyme on the electrodes has an important influence on the rate of the cell reaction. It is necessary, therefore, to estimate as closely as possible how much free energy is liberated when an enzyme molecule is adsorbed on the electrode. From this, the number of molecules adsorbed per cm^2 can be deduced.

Three factors contribute to the adsorption energy:

(1) Van der Waal's forces

(2) "Image" forces due to dipoles in the enzyme molecule

(3) Hydrogen bonds between oxide or hydroxyl on the metal surface and oxygen or nitrogen containing groups of the enzyme adjacent to the electrode.

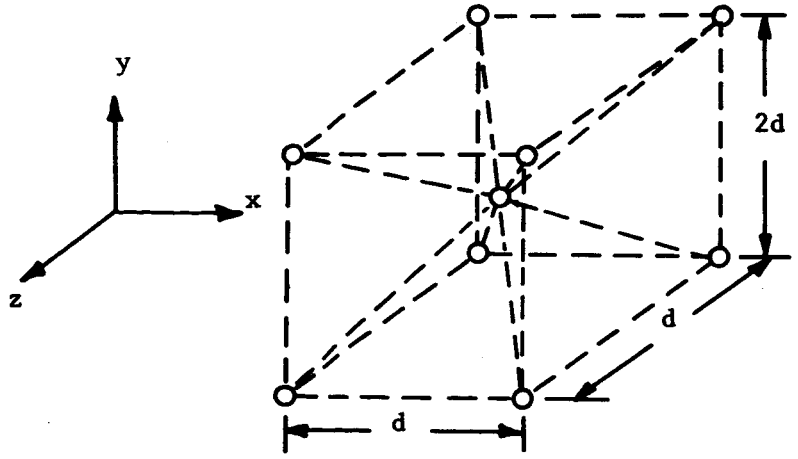
Only the last need be considered, since it is known to account for 95-98 percent of the binding energy in similar systems.

It is known that the molecular weight of the enzyme is approximately 100,000, that the molecules are spheres 60-65 Å in diameter, and that the protein part of the molecule is very similar to myoglobin in its composition and properties.

The composition, and the conclusions to be drawn from it, are shown in Table 8. The line of reasoning on which the conclusions are based must be outlined.

First of all, the relative abundance of the various amino acid residues is shown in column 3. The molecular weight, 100,000, divided by the average molecular weight per residue gives the number of residues per molecule--890. The arrangement of the component parts for myoglobin is known from X-ray studies; they appear in a convoluted helical chain (and in repeating sequence). This complex pattern is quite unsuitable for the present analysis. Instead, a close-packed, randomly arranged array was assumed, subject only to the restriction that the number of amino acids of each kind is to be proportional to its relative frequency (column 3).

The unit cell of such a close-packed array (cubic) is as shown below.



Its volume is $\sqrt{2}\ d^3$, and it contains 2 groups (one in the center, 8 at the corners, each shared equally by 8 unit cells). In the x and y directions the area is $\sqrt{2}\ d^2$, and one group occupies this area (4 at the corners, each shared by 4 such areas). The surface density ρ_x and ρ_y is $1/\sqrt{2}d^2$ groups per cm^2. In the z direction one group occupies d^2 cm^2, so $\rho_z = 1/d^2$. These three surface densities can be considered as the semiaxes of an ellipsoid of revolution, which has the useful property that the surface density in any direction is equal to the distance from the center in that direction. It follows (the proof is omitted) that the average surface density of a sphere composed of units like that shown is equal to the radius of a sphere whose volume is equal to that of the ellipsoid:

$$\frac{4}{3}\ \bar{\rho}^3 = \frac{4}{3}\pi\rho_x\ \rho_y\ \rho_z = \frac{4}{3}\pi\ \frac{1}{\sqrt{2}d^2}\ \frac{1}{\sqrt{2}d^2}\ \frac{1}{d^2}$$

$$\bar{\rho} = \frac{1}{\sqrt[3]{2}d^2}$$

From this, the number of amino acid groups at the surface of the enzyme molecule can be determined. The radius of the molecule is R, its surface is $4\pi R^2$, and the number of surface groups, n_s, is:

$$n_s = 4\pi R^2\ \bar{\rho} = \frac{4\pi R^2}{\sqrt[3]{2}d^2}$$

Its volume is $4/3\pi R^3$, so

$$n_{total} = \frac{\frac{4}{3}\pi R^3}{\left(\frac{\text{Volume}}{\text{group}}\right)} = \frac{\frac{4}{3}\pi R^3}{\frac{\sqrt{2}}{2}\ d^3} = 890$$

Combining these,

$$n_s = \sqrt[3]{9\pi}\,(n_{total})^{2/3} = 232$$

This, multiplied by the number fraction of each kind of amino acid gives the number of each at the surface (column 6). The oxygen- and nitrogen-containing groups to form the hydrogen bonds are listed in column 4.

Evidently, not all these groups will bond to this surface; most will be too far away (hydrogen bonds cannot span distances greater than about 2 Å), and some of those close enough will have already been bonded to other groups within the enzyme.

The fraction which is close enough is simply the ratio

$$\frac{\text{(surface of the sphere within 2 Å of the electrode)}}{\text{(total surface of the sphere)}}, \text{ or}$$

$$f_{<2\text{Å}} = \frac{2\pi R \cdot 2\text{Å}}{4\pi R^2} = \frac{1}{R} = 0.0320$$

The fraction not already bonded is not quite so obvious. In the strongly hydrated and randomly oriented (in this model) enzyme protein, a group can form a hydrogen bond in any direction. Whether it does form one will depend on its opportunities for contact with another bondable group---i.e., on how many nearest neighbors it has. An interior group has 12, and a surface group only 9, with the electrode surface taking the place of the other three. Presumably, then, one-fourth of the surface groups within 2 Å may actually bond to the surface.

To sum up, 0.8 percent of the surface groups (i.e., 0.032 x 0.25) should form hydrogen bonds to the electrode. To determine the adsorption energy, multiply the number of surface groups (column 6 in Table 1) by 0.008, sort out the different kinds of bonds formed (e.g., C=O....HO Metal, or N....H-O-Metal), and multiply the number of each kind by the appropriate energy per bond. Fortunately, this last is well known (L. Pauling, The Nature of the Chemical Bond, pp 31, 284ff).

Bond	Kcal/mole
C=O----H O —	7.0
N H----O ——	5.2
N----H O —	3.8
C=N H----O —	6.7

Since entropy changes cannot be calculated for these reactions, ΔF must be assumed to differ (only slightly) from ΔH. The final result of this line of reasoning suggests that adsorption of an enzyme molecule on the electrode releases about 35.1 kcal per mole of free energy. This is much higher than the energy release for most adsorption processes, and should be interpreted quantitatively.

$$\Delta F^{o}\ (35.1\ \text{kcal/mole}) = -RT \ln K_{\text{equilibrium}}, \text{ where}$$

$$K_{\text{equilibrium}} = \frac{\text{activity of adsorbed molecules}}{\text{activity in solution}} \simeq \frac{C_a}{C_s}$$

$$C_a = C_s\, e^{-\frac{\Delta F^{o}}{RT}} = C_s\, e^{+\frac{35100}{600}} \quad \text{at } 25^{o}\ C.$$

$$= C_s\, e^{58.5}$$

The ratio of C_a/C_s is, obviously, a very large number under these conditions and the concentration of adsorbed molecules would be many orders of magnitude higher than the surface can accommodate even at very low solution concentration since the capacity of the surface would be 5×10^{-3} M (close packed layer). At least a ten fold reduction in the estimated ΔF for adsorption would be required to obtain realistic values for C_a/C_s. A more detailed treatment may therefore postulate a close-packed monolayer at the surface, and a plurality of more loosely packed layers at greater distances. But for the present purpose the single close packed layer is adequate; it can be shown that additional layers have little further effect on reaction rates.

7.2 APPLICATION TO REACTION RATES IN THE CELL

It will be convenient to analyze electrode reaction rates according to the following schemes:

A. Enzyme-substrate reaction takes place in the body of the solution.

   (1) The rate is determined by the reactivity of the enzyme.

   (2) The rate is determined by diffusion of the enzyme.

   (3) The rate is determined by the exchange rate of oxidized and reduced enzyme at the anode.

   (4) The rate is determined by the pH of the solution.

B. Enzyme-substrate reaction takes place at the anode.

(1) The rate is determined by diffusion of substrate.

(2) The rate is determined by the number of properly oriented enzyme molecules adsorbed.

Since the enzyme functions strictly as a catalyst, it might appear at first sight that its relatively slow reaction time ($\sim$ 16 per second) would slow up the overall reaction. For the same reaction (oxidation of amino acid to pyruvic acid) conducted under other conditions this might be true. If, for instance, the reduced enzyme were to be reoxidized by dissolved oxygen, with almost unlimited possibilities for contact between enzyme and oxidant, increased catalytic activity would certainly increase the rate. But in the fuel cell, any reoxidation except at the anode is by definition an undesirable side reaction and a waste of fuel. The effectiveness of the enzyme is governed primarily by the rate at which it participates in the anode reaction, or by the rate of its transfer to the anode. Its ability, in the oxidized form, to react rapidly with substrate can limit the rate only if the time scale for this process (0.06 second) can be shown to be large in comparison with the time scale of the anode process and of the transfer process. To settle this point, both processes must be considered in detail.

First, the diffusion. To study this, we consider that the cell has reached a steady state, without specifying what current is being drawn. A characteristic property of such a steady state is that the concentration of every species within a given element of volume remains constant. The gradual depletion of substrate can be ignored, as of no effect, or corrected for by continuous addition.

Any reacting species can be singled out, and its constancy of concentration analyzed in terms of processes which will increase or decrease that concentration. The rate of all such processes must add up to zero. For convenience, the oxidized enzyme was chosen for analysis. The volume considered is a sandwich-shaped element parallel to the anode, 1 cm on a side, and Δcm thick. Three processes can change the concentration within this volume, as represented in Figure 43 which shows concentration of oxidized enzyme versus distance from the anode. First, enzyme will diffuse in at a rate $\mathcal{D}\,(dc/dx)_x$, where $\mathcal{D}$ is the diffusion constant, 6.8×10^{-7} cm^2 sec^{-1}. Second, enzyme can diffuse out through the opposite boundary at a rate $\mathcal{D}\,(dc/dx)_{x+\Delta x}$. Third, enzyme within the element can disappear by reacting with substrate, the rate being kC, when k is the rate constant for oxidation, (k = 1000 min^{-1} or 16 sec^{-1}). The thickness, Δx, can be decreased as far as we please without disturbing our imposed condition of constant concentration of enzymes. Accordingly, the sum of rates,

$$+\mathcal{D}\left(\frac{dc}{dx}\right)_x - kc - \mathcal{D}\left(\frac{dc}{dx}\right)_{x+\Delta x} = 0$$

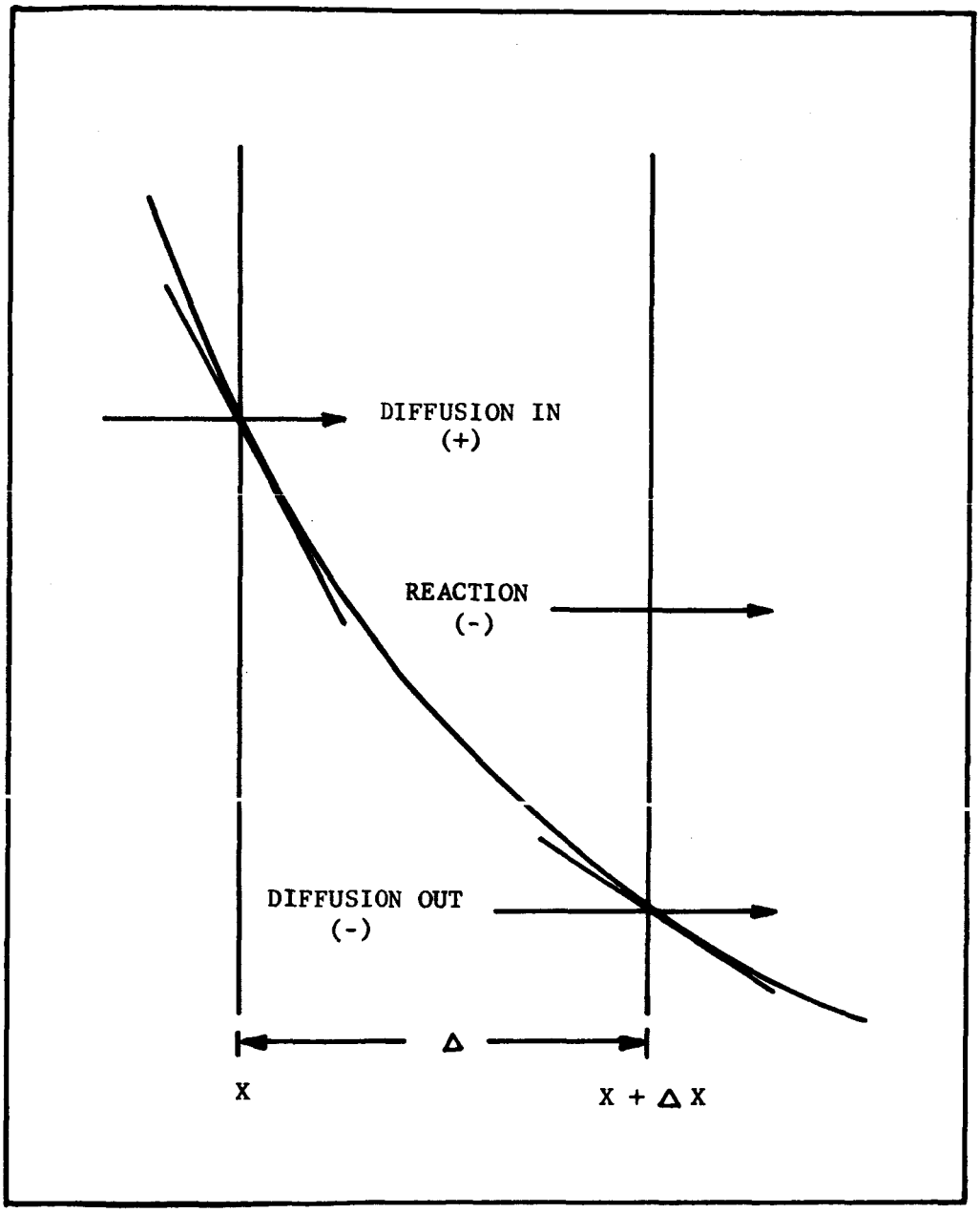


FIGURE 43. DIFFUSION MODEL FOR THEORECTICAL CONSIDERATIONS

can be simplified as $\Delta x \rightarrow 0$:

$$\mathcal{D}\left(\frac{d^2c}{dx^2}\right) = kc.$$

The solution is obvious:

$$c = A\, e^{-\sqrt{\frac{k}{\mathcal{D}}}\, x} + B\, e^{+\sqrt{\frac{k}{\mathcal{D}}}\, x}$$

The B term must be zero, since the concentration cannot increase as the distance from the anode increases (diffusion would then take place in the wrong direction). The proper equation,

$$c = c_{anode}\, e^{-\sqrt{\frac{k}{\mathcal{D}}}\, x}$$

must hold everywhere, since our volume element was randomly chosen. Accordingly, it must also hold at the surface of the anode. But at that point $-\mathcal{D}\,(dc/dx)_{x=0}$ must be equal to the number of oxidized enzyme molecules diffusing away from the anode, which in turn is equal to the rate of the overall reaction, since oxidation cannot take place anywhere else. That is,

$$n = -\mathcal{D}\left(\frac{dc}{dx}\right)_{x=0} = c_{anode}\sqrt{k\mathcal{D}}$$

$\mathcal{D}$ and k are known already. C_{anode} is known in two dimensions -- 2.956×10^{12} molecules per cm^2 for a close-packed layer. If the anode layer is considered to extend in the X-direction only to the diameter of the adsorbed molecules (62.5 Å),

$$c_{anode} = \frac{2.958 \times 10^{12}}{(6.25 \times 10^{-7})\,(6.023 \times 10^{23})} \text{ moles per cm}^3.$$

$$= 7.8528 \times 10^{-6} \text{ moles/cm}^3.$$

From this the steady-state current i can be determined:

$$i = 2 \mathcal{F} c_o \sqrt{k \mathcal{D}}, \text{ or}$$

$$i = 4.999 \times 10^{-3} \text{ amperes per cm}^2.$$

It must be borne in mind that this "i" is defined on a purely hypothetical basis -- that the cell current is diffusion-limited, which has not been proved. But its significance should not be overlooked; unless further analysis uncovers some even slower rate-determining process, the cell can obviously generate no more than 5 milliamps per cm^2, a very modest level of performance, which can only be revised downward.

The anode process itself, rather than the enzyme's diffusion away from the anode, may be the rate-determining step. One part of this process -- the transfer of charge to the anode -- cannot be analyzed even qualitatively, since no data even remotely relevant are on record. This omission becomes serious only if other, analyzable, processes are fast enough to suggest that charge transfer is the rate determining step. This will be considered again, in the summary, when it can be fitted into context.

The anode process can be split into three subprocesses, as shown below and in Figure 43.

$$\text{(reduced enzyme)}_{\text{solution}} \xrightarrow{\text{I}} \text{(reduced enzyme)}_{\text{adsorbed}}$$

$$\text{(reduced enzyme)}_{\text{adsorbed}} \xrightarrow{\text{II}} \text{(oxidized enzyme)}_{\text{adsorbed}} + 2e^-$$

$$\text{(oxidized enzyme)}_{\text{adsorbed}} \xrightarrow{\text{III}} \text{(oxidized enzyme)}_{\text{solution}}$$

The rate of adsorption of reduced enzyme, depends upon the concentration gradient at the surface, which depends upon the number of available sites on which reduced enzymes can be adsorbed, which in turn depends on the rate of desorption of oxidized enzyme. II, as noted above, is unanalyzable. Thus, one point of attack is III, the rate at which oxidized enzyme is desorbed.

Process III can be analyzed in terms of the free energy required, which consists of the adsorption energy, 35.1 kcal/mole minus the free energy released when the molecule is transferred from the high concentration at the anode to the lower concentration in the solution. The latter term is

$$\Delta F_{\text{concentration}} = -RT \ln \frac{C_{\text{anode}}}{C_{\text{solution}}} = -2.56 \text{ kcal/mole}$$

The energy which must be made available to the enzyme molecule if it is to desorb is therefore 32.54 kcal/mole.

There is only one possible source for this energy -- the kinetic energy of solvent molecules, which can be transferred on impact. The rate of desorption (dn/dt) is therefore the number of adsorbed enzyme molecules which are struck, per cm^2 per second, by solvent molecules whose kinetic energy is equal to or greater than the 32.54 kcal/mole required for desorption.

$$\left(\frac{dn}{dt}\right) = \text{(number of solvent molecules striking 1 cm}^2\text{ per second)}$$

$$f = \text{(fraction where kinetic energy is } \geq 32.54 \text{ kcal/mole).}$$

Neither (dn/dt) nor f can be determined for liquids. To avoid this difficulty, desorption was considered to take place by means of impacts of molecules of a high pressure gas. The rate of desorption for this analogous system can be deduced as a function of the pressure, the molecular weight, and the temperatures. If a high enough value is assigned the pressure, we can be certain that the desorption rate in the real (liquid) system will be less than that for the gas system.

On this basis,

$$\left(\frac{dn}{dt}\right) = \frac{p}{\sqrt{2\pi mkT}} = \frac{N_{op}}{\sqrt{2\pi MRT}}$$

where p = pressure in dynes/cm^2, and M = molecular weight (18.016). The second term can be evaluated by use of the Maxwell-Boltzmann distribution law:

$$p\ (v) = 4\pi \left(\frac{m}{2\pi kT}\right)^{3/2} v^2\, e^{-\frac{mv^2}{2kT}}\, dv$$

or more conveniently its dimensionless form, with $x = E_k/RT$

$$p\ (x) = \frac{2}{\sqrt{\pi}}\ \sqrt{x}\, e^{-x}\, dx$$

where $X = \Delta E_{kinetic}/RT$. The probability that the kinetic energy will equal or exceed E_k is

$$p\ (\geq x) = \frac{2}{\sqrt{\pi}} \int_{\frac{\Delta E_k}{RT}}^{\infty} \sqrt{x}\, e^{-x}\, dx = \frac{2}{\sqrt{\pi}}\ \sqrt{x+1}\ e^{-x}$$

As would be expected, this fraction is exceedingly small, 1.155×10^{-23}.

The desorption rate (this fraction x 2.357×10^{27}, the number striking cm^2 per second) is

$$\left(\frac{dn}{dt}\right) = 2.723 \times 10^4 \ p_{atm}$$

It is not obvious what value should be assigned p in the case of a liquid, but the choice is not of crucial importance. Even if p is set equal to 10^4 atmospheres, surely a gross overestimate, no more than 2.723×10^8 enzyme molecules can be desorbed per cm^2 per second. The desorption-limited current is

$$i \leq \frac{2\mathcal{F}}{n_o} \left(\frac{dn}{dx}\right) = 8.725 \times 10^{-9} \text{ amperes/cm}^2.$$

Currents this small can be measured, provided the galvanometer is sensitive enough, but they are unlikely to serve any very useful purpose.

Essentially, the low reaction rate is due to the high adsorption energy. This can be significantly lowered by decreasing the pH of the electrolyte; ionization of carboxyls is inhibited and ionization of amino groups is enhanced, leading to a net positive charge on the enzyme molecule. Electrostatic repulsion between positively charged enzyme and positively charged anode can in principle, at low enough pH's, decrease the adsorption energy to the point when desorption is no longer the rate-determining step. In practice, however, the efficiency of the cell will not be increased, for the following reasons: (a) the enzyme is a less active catalyst in acid solutions, (b) reduced enzyme will migrate to the cathode and form a high-resistance layer, (c) diffusion of reduced enzyme to the anode will be slowed up by electrostatic repulsion.

It must be concluded, then, that if the enzyme-substrate reaction takes place in the body of the solution, a cell involving electrochemical oxidation of the enzyme can produce no more than about 5 milliamperes per cm^2.

On the other hand, it is quite possible that the enzyme adsorbed on the anode can oxidize the substrate and be reoxidized itself without having to desorb and diffuse anywhere else. Diffusion of the substrate to the anode should be rapid enough to support sizeable currents.

Not all adsorbed molecules can take part in the reaction, however. Most of them will be so oriented that the side chain containing the prosthetic group (10.6 Å in length) cannot come in contact with the electrode. Charge transfer must then be either by conduction through the protein, or by some high-resistance ionic path around it. Conduction through proteins is known to occur, particularly when they are heavily solvated, as the enzyme is; they function as semiconductors whose conduction band is about two electron volts above the valence band. Accordingly, the resistance is so high that conduction must be ruled out as a feasible mechanism for charge transfer.

The fraction of the adsorbed enzyme molecules which are oriented to allow charge transfer by direct contact between prosthetic group and anode is

$$f_{\text{direct contact}} = \frac{(\text{area on sphere within } 10.6 \text{ Å of surface})}{(\text{total area of sphere})}$$

$$= \frac{(2\pi)\,(31.25)\,(10.6)}{4\pi\,(31.25)^2} = 0.1696$$

The corresponding reaction rate is

$$\left(\frac{dn}{dt}\right) = (f_{\text{direct contact}}) \left(\frac{\text{enzyme molecules adsorbed}}{\text{cm}^2}\right) \left(\frac{\text{reaction rate}}{\text{molecule}}\right)$$

$$= (0.1696)\,(2.956 \times 10^{12})\,(16) = 8.00 \times 10^{12} \text{ per second}$$

and the current

$$i = \frac{2\mathcal{F}}{n_o} \left(\frac{dn}{dt}\right) = 2.571 \times 10^{-6} \text{ amperes/cm}^2.$$

These conclusions would probably be valid for any enzyme reaction although slight improvements might be made were the enzyme to be smaller with a higher diffusion constant, smaller diameter, and with a higher turnover number. However, the diffusion constant will not vary by more than one magnitude for normal enzymes; decreases in size and volume will not permit an adequate increase in number of adsorbed molecules; reaction velocity (for the enzyme-substrate reaction) has entered into the problem only in the case of the enzyme substrate reaction occurring at the electrode with adsorbed enzyme. Since the most rapid enzyme reaction known (catalase) only gives a turnover of 10^7 reactions/molecule/min (a factor of 10^4 greater than the figures used here) maximum currents would still be of too low an order (max. $\sim$ 20 ma) to provide a good power source.

SECTION 8

GENERAL CONCLUSIONS AND SUMMARY

The initial impetus for undertaking the present bioelectrode research program was the need to obtain basic information on the operation of the bioelectrode to support development of a practical biofuel cell. It became obvious, during the course of work at Philco Research Laboratories and elsewhere, that many more fundamental problems existed in the development of a bioelectrode, particularly on the nature of the electrode reaction, than had been anticipated at the outset. Accordingly, the accent in the present research has been on the elucidation of the electrode reactions of various model systems which could be expected to occur in the biological oxidations of organic materials. Of particular concern was the function of the enzyme in the electrode reaction. Certain areas of research, more directly concerned with specific applications, were, of necessity, given less attention in order to devote time to the more fundamental problems. Thus, since the questions of interaction of cell and electrode, in the attached organism bioelectrode, were much more extensive than visualized, these interactions were studied while little attention was given to the problems of viability or even the best means of attachment.

The studies on electrode reactions at the enzymatic level indicate that only two types of systems may actually function for practical purposes in the bioelectrode. These are: the reactions which will produce, through enzymatic conversion, an electroactive product from an electrochemically inactive fuel; and reactions in which a mediator is used to carry electrons from the enzyme to the electrode after the enzyme has been reduced by reaction with the fuel material. The present experimental studies, together with some theoretical considerations, seem to exclude the other possibility of direct reaction of an enzyme with the electrode. Thus, it is possible to obtain reasonably efficient reactions where a carrier molecule is used to oxidize reduced glucose oxidase, amino acid oxidase, mitochondria, or any of a number of other enzyme systems, and the carrier is subsequently oxidized electrochemically to act as a regenerative mediator. It is also possible to form electroactive products from inactive materials, as in forming IPA

from tryptophan or hydroxylamine from nitrite, or hydrogen sulfide from sulfite, to obtain electrochemical reactions. However, tests of enzymes such as DAO at substrate levels indicate that there is either no reaction of such enzymes with the electrode or such reaction is so slow as to be impractical. Theoretical calculations of interactions between model enzymes and the electrode support the conclusion that such reactions would produce vanishingly low currents.

The investigation of some systems which were purported to be of significant value in bioelectrode applications, e.g., the urease-urea reaction, revealed entirely different sources of electroactivity from those which had been assumed to be responsible. Thus, in the system cited, currents observed as a result of the action of urease on urea in the electrochemical cell were proven to be the result of pH activation of oxidizable groups in impurities of the enzyme preparations. Since bacterial systems which hydrolyze urea are capable of producing reasonable amounts of currents over a long period of time, (22) it would appear that such reactions should be investigated with a view toward establishing, with certainty, the products responsible for the observed currents.

Experiments with electrode attached enzymes and organisms using simple agar supporting media seemed to demonstrate some advantages of the attached catalysts over the free solution catalysts on the basis of rate of development of activity and the power obtained per unit of enzyme or organism. However, calculations based upon equivalent volumes of enzyme solution or the attached, immobilized enzyme (or organism) indicate that the bulk medium catalyst may be the more efficient system by as much as two orders of magnitude. Much more work with more refined systems will be required, however, to determine whether sufficiently thin and properly organized, immobilized layers might be the more efficient. It must be recognized here, that the immobilized systems now being considered are those in which the advantages of attachment would accrue through improvements in diffusional relations with reactants and products and not through the direct reaction of electrode and organism or enzyme.

While the work cited above definitely minimizes any possibility of obtaining an advantage in electrochemical activity through the direct interaction of a simple enzyme with the electrode, this must not be taken to exclude, completely, theoretical improvements due to organized structures. Certainly it is possible to visualize systems in which potential gradients across immobilized layers of the polyelectrolyte material composing an organism, would act to speed diffusion of reactants to the electrode and products away. It is also possible to conceive that the ordered structure of the cell would act as a finger or arm reaching into the medium in such a manner as to trap the fuel and, through the efficient organization of the cell, transmit electrons to the electrode from reactions occurring at some distance from the electrode surface. However, such reactions remain to be investigated and cannot be invoked at this time as support for immobilized bioelectrode systems.

Thus, it is obvious that many fundamental areas of interaction between biological catalysts and electrochemical systems remain to be explored in order to obtain fully efficient and predictable bioelectrodes.

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